DKT. 9643-1/SBA

CTLA4 MOLECULES AND IL4-BINDING MOLECULES AND USES THEREOF

This application is a continuation-in-part of U.S. Serial No. 08/008,898, filed January 22, 1993, which is a continuation-in-part of U.S. Serial No. 723,617, filed July 27, 1991, now abandoned, the contents of all of which are incorporated by reference into the present application.

Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The present invention relates to expression of CTLA4 hybrid fusion proteins, the CTLA4 receptor gene, identification of the interaction between the CTLA4 receptor and cells expressing B7 antigen, and to methods for regulating cellular interactions involving the CTLA4 receptor and the B7 antigen.

BACKGROUND OF THE INVENTION

The hallmark of a vertebrate immune system is the ability to discriminate "self" from "non-self" (foreign). This property has led to the evolution of a system requiring multiple signals to achieve optimal immune activation (Janeway, Cold Spring Harbor Symp. Quant. Biol. 54:1-14 (1989)). T cell-B cell interactions are essential to the immune response. Levels of many cohesive molecules found on T cells and B cells increase during an immune response (Springer et al., (1987), supra; Shaw and Shimuzu, Current Opinion in Immunology, Eds. Kindt and Long, 1:92-97 (1988)); and Hemler Immunology Today 9:109-113 (1988)). Increased levels of these molecules may help explain why activated B cells are more

effective at stimulating antigen-specific T cell proliferation than are resting B cells (Kaiuchi et al., J. Immunol. 131:109-114 (1983); Kreiger et al., J. Immunol. 135:2937-2945 (1985); McKenzie, J. Immunol. 141:2907-2911 (1988); and Hawrylowicz and Unanue, J. Immunol. 141:4083-4088 (1988)).

The generation of a T lymphocyte ("T cell") immune response is a complex process involving cell-cell interactions (Springer et al., A. Rev. Immunol. 5:223-252 (1987)), particularly between T and accessory cells such as B cells, and production of soluble immune mediators (cytokines or lymphokines) (Dinarello and Mier, New Engl. Jour. Med 317:940-945 (1987)). This response is regulated by several T-cell surface receptors, including the T-cell receptor complex (Weiss et al., Ann. Rev. Immunol. 4:593-619 (1986)) and other "accessory" surface molecules (Springer et al., (1987) supra). Many of these accessory molecules are naturally occurring cell surface differentiation (CD) antigens defined by the reactivity of monoclonal antibodies on the surface of cells (McMichael, Ed., Leukocyte Typing III, Oxford Univ. Press, Oxford, N.Y. (1987)).

Antigen-independent intercellular interactions involving lymphocyte accessory molecules are essential for an immune response (Springer et al., (1987), supra). For example, binding of the T cell-associated protein, CD2, to its ligand LFA-3, a widely expressed glycoprotein (reviewed in Shaw and Shimuzu, supra), is important for optimizing antigen-specific T cell activation (Moingeon et al., Nature 339:314 (1988)).

An important adhesion system involves binding of the LFA-1 glycoprotein found on lymphocytes, macrophages, and granulocytes (Springer et al., (1987), supra; Shaw and Shimuzu (1988), supra) to its ligands ICAM-1 (Makgoba et al., Nature 331:86-88 (1988)) and ICAM-2 (Staunton et al., Nature 339:61-64 (1989)). The T cell accessory molecules CD8 and CD4 strengthen T cell adhesion by

interaction with MHC class I (Norment et al.,Nature 336:79-81 (1988)) and class II (Doyle and Strominger, Nature 330:256-259 (1987)) molecules, respectively. "Homing receptors" are important for control of lymphocyte migration (Stoolman, Cell 56:907-910 (1989)).

The VLA glycoproteins are integrins which appear to mediate lymphocyte functions requiring adhesion to extracellular matrix components (Hemler, supra). The CD2/LFA-3, LFA-1/ICAM-1 and ICAM-2, and VLA adhesion systems are distributed on a wide variety of cell types (Springer et al., (1987), supra; Shaw and Shimuzu, (1988,) supra and Hemler, (1988), supra).

Numerous in vitro studies have demonstrated that cytokines are involved in the generation of alloreactive effector cells. For example, membrane bound IL-4 and soluble IL-4 receptor were administered separately to mice and were shown to augment the lymphoproliferative response (William C. Fanslow et al. "Regulation of Alloreactivity in vivo by IL-4 and the soluble Il-4 receptor" J. Immunol. 147:535-540 (1991)). Specifically, administration of IL-4 to BALB\c mice resulted in slight augmentation of the lymphoproliferative response. In contrast, the soluble IL-4 receptor suppressed this response to allogeneic cells in a dose dependent manner. Moreover, a neutralizing antibody against IL-4 and another against soluble IL-4 receptor were effective inhibitors of the lymphoproliferative response.

It was proposed many years ago that B lymphocyte activation requires two signals (Bretscher and Cohn, Science 169:1042-1049 (1970)) and now it is believed that all lymphocytes require two signals for their optimal activation, an antigen specific or clonal signal, as well as a second, antigen non-specific signal (Janeway, supra). Freeman et al. (J. Immunol. 143(8):2714-2722 (1989)) isolated and sequenced a cDNA clone encoding a B cell activation antigen recognized by mAb B7 (Freeman et al., J. Immunol. 138:3260

(1987)). COS cells transfected with this cDNA have been shown to stain by both labeled mAb B7 and mAb BB-1 (Clark et al., Human Immunol. 16:100-113 (1986); Yokochi et al., J. Immunol. 128:823 (1981)); Freeman et al., (1989) supra; and Freedman et al., (1987), supra)). In addition, expression of this antigen has been detected on cells of other lineages, such as monocytes (Freeman et al., supra).

The signals required for a T helper cell (T_h) antigenic response are provided by antigen-presenting cells (APC). The first signal is initiated by interaction of the T cell receptor complex (Weiss, J. Clin. Invest. 86:1015 (1990)) with antigen presented in the context of class II major histocompatibility complex (MHC) molecules on the APC (Allen, Immunol. Today 8:270 (1987)). This antigen-specific signal is not sufficient to generate a full response, and in the absence of a second signal may actually lead to clonal inactivation or anergy (Schwartz, Science 248:1349 (1990)). The requirement for a second "costimulatory" signal provided by the MHC has been demonstrated in a number of experimental systems (Schwartz, supra; Weaver and Unanue, Immunol. Today 11:49 (1990)). The molecular nature of this second signal(s) is not completely understood, although it is clear in some cases that both soluble molecules such as interleukin (IL)-1 (Weaver and Unanue, supra) and membrane receptors involved in intercellular adhesion (Springer, Nature 346:425 (1990)) can provide costimulatory signals.

CD28 antigen, a homodimeric glycoprotein of the immunoglobulin superfamily (Aruffo and Seed, Proc. Natl. Acad. Sci. 84:8573-8577 (1987)), is an accessory molecule found on most mature human T cells (Damle et al., J. Immunol. 131:2296-2300 (1983)). Current evidence suggests that this molecule functions in an alternative T cell activation pathway distinct from that initiated by the T-cell receptor complex (June et al., Mol. Cell. Biol. 7:4472-4481 (1987)). Monoclonal antibodies (mAbs) reactive with CD28 antigen

can augment T cell responses initiated by various polyclonal stimuli (reviewed by June et al., supra). These stimulatory effects may result from mAb-induced cytokine production (Thompson et al., Proc. Natl. Acad. Sci 86:1333-1337 (1989); and Lindsten et al., Science 244:339-343 (1989)) as a consequence of increased mRNA stabilization (Lindsten et al., (1989), supra). Anti-CD28 mAbs can also have inhibitory effects, i.e., they can block autologous mixed lymphocyte reactions (Damle et al., Proc. Natl. Acad. Sci. 78:5096-6001 (1981)) and activation of antigen-specific T cell clones (Lesslauer et al., Eur. J. Immunol. 16:1289-1296 (1986)).

Studies have shown that CD28 is a counter-receptor for the B cell activation antigen, B7/BB-1 (Linsley et al, Proc. Natl. Acad. Sci. USA 87:5031-5035 (1990)). For convenience the B7/BB-1 antigen is hereafter referred to as the "B7 antigen". The B7 ligands are also members of the immunoglobulin superfamily but have, in contrast to CD28 and CTLA4, two Ig domains in their extracellular region, an N-terminal variable (V)-like domain followed by a constant (C)-like domain.

An important non-specific costimulatory signal is delivered to the T cell when there are at least two homologous B7 family members found on APC's, B7-1 (also called B7 or CD80) and B7-2, both of which can deliver costimulatory signals to T cells via either CD28 or CTLA4. Costimulation through CD28 or CTLA4 is essential for T cell activation since a soluble Ig fusion protein of CTLA4 (CTLA4-Ig) has successfully been used to block T cell activation events *in vitro* and *in vivo*. Failure to deliver this second signal may lead to clonal inactivation or T cell anergy.

Interactions between CD28 and B7 antigen have been characterized using genetic fusions of the extracellular portions of B7 antigen and CD28 receptor, and Immunoglobulin (Ig) Cγ1 (constant region heavy chains) (Linsley et al, J. Exp. Med. 173:721-730 (1991)). Immobilized B7Ig fusion protein, as well as B7 positive CHO cells,

have been shown to costimulate T cell proliferation.

T cell stimulation with B7 positive CHO cells also specifically stimulates increased levels of transcripts for IL-2. Additional studies have shown that anti-CD28 mAb inhibited IL-2 production induced in certain T cell leukemia cell lines by cellular interactions with a B cell leukemia line (Kohno et al., Cell. Immunol. 131-1-10 (1990)).

CD28 has a single extracellular variable region (V)-like domain (Aruffo and Seed, supra). A homologous molecule, CTLA4 has been identified by differential screening of a murine cytolytic-T cell cDNA library (Brunet et al., Nature 328:267-270 (1987)).

Transcripts of the CTLA4 molecule have been found in T cell populations having cytotoxic activity, suggesting that CTLA4 might function in the cytolytic response (Brunet et al., supra; and Brunet et al., Immunol. Rev. 103-21-36 (1988)). Researchers have reported the cloning and mapping of a gene for the human counterpart of CTLA4 (Dariavach et al., Eur. J. Immunol. 18:1901-1905 (1988)) to the same chromosomal region (2q33-34) as CD28 (Lafage-Pochitaloff et al., Immunogenetics 31:198-201 (1990)). An Ig fusion of CTLA4 binds to B7-1 with ≈20 fold higher avidity than a corresponding Ig fusion of CD28.

Sequence comparison between this human CTLA4 DNA and that encoding CD28 proteins reveals significant homology of sequence, with the greatest degree of homology in the juxtamembrane and cytoplasmic regions (Brunet et al., 1988, supra; Dariavach et al., 1988, supra).

The high degree of homology between CD28 and CTLA4, together with the co-localization of their genes, raises questions as to whether these molecules are also functionally related. However, since the protein product of CTLA4 has not yet been successfully expressed,

these questions remain unanswered.

Expression of soluble derivatives of cell-surface glycoproteins in the immunoglobulin gene superfamily has been achieved for CD4, the receptor for HIV-1, and CD28 and B7 receptors, using hybrid fusion molecules consisting of DNA sequences encoding amino acids corresponding to portions of the extracellular domain of CD4 receptor fused to antibody domains (immunoglobulin γ1 (Capon et al., Nature 337:525-531 (1989) (CD4) and Linsley et al., J. Exp. Med., supra (CD28 and B7)).

There is a need for molecules which can identify in vitro B7 positive B cells, i.e., activated B cells, for leukocyte typing and FAC sorting. Further, there is a need for molecules which may be used to prevent the rejection of organ transplants and inhibit the symptoms associated with lupus erythmatosus and other autoimmune diseases. In the past, major therapies relied on panimmunosuppressive drugs, such as cyclosporine A or monoclonal antibodies (MAbs) to CD3 to prevent organ transplants or inhibit symptoms of lupus. Unfortunately, these drugs must frequently be taken for the life of the individual, depress the entire immune system, and often produce secondary health ailments such as increased frequency of infections and cancer.

SUMMARY OF THE INVENTION

Accordingly, the present invention provides the complete and correct DNA sequence encoding the amino acid sequence corresponding to the CTLA4 receptor protein, and identifies B7 antigen (e.g. B7-1 and B7-2 antigens) as a natural ligand for the CTLA4 receptor. The invention also provides a method for expressing the DNA as a CTLA4 immunoglobulin (Ig) fusion protein product. Embodiments of the invention include CTLA4Ig fusion protein, and hybrid fusion proteins including CD28/CTLA4Ig fusion proteins (which is also referred to herein as the CTLA4/CD28Ig fusion protein). Also

provided are methods for using the CTLA4 fusion protein, B7Ig fusion protein, hybrid fusion proteins, and fragments and/or derivatives thereof, such as monoclonal antibodies reactive with CTLA4 and the B7 antigen, to regulate cellular interactions and immune responses.

The human CTLA receptor protein of the invention is encoded by 187 amino acids and includes a newly identified N-linked glycosylation site.

The CTLA4Ig fusion protein of the invention binds the B7 antigen expressed on activated B cells, and cells of other lineages, a ligand for CD28 receptor on T cells. The CTLA4Ig binds B7 antigen with significantly higher affinity than B7 binding to the CD28 receptor. The CTLA4Ig construct has a first amino acid sequence corresponding to the extracellular domain of the CTLA4 receptor fused to a second amino acid sequence corresponding to the human Ig Cγ1 domain. The first amino acid sequence contains amino acid residues from about position 1 to about position 125 of the amino acid sequence corresponding to the extracellular domain of CTLA4 joined to a second amino acid sequence containing amino acid residues corresponding to the hinge, CH2 and CH3 regions of human IgCγ1. The fusion protein is preferably produced in dimeric form. Soluble CTLA4Ig is a potent inhibitor <u>in vitro</u> of T and B lymphocyte responses.

Also contemplated in the invention are soluble CTLA4 and hybrid fusion proteins thereof, e.g., soluble hybrid fusion proteins, such as CD28/CTLA4Ig fusion proteins. The extracellular domain of CTLA4 is an example of a soluble CTLA4 molecule. Alternatively, a molecule having the extracellular domain of CTLA4 attached to a peptide tag is another example of a soluble CTLA4 molecule.

As an example of a soluble hybrid fusion protein, the present invention provides CD28/CTLA4Ig fusion proteins having a first

amino acid sequence corresponding to fragments of the extracellular domain of CD28 joined to a second amino acid sequence corresponding to fragments of the extracellular domain of CTLA4Ig and a third amino acid sequence corresponding to the hinge, CH2 and CH3 regions of human IgCγ1. One embodiment of the hybrid fusion proteins is a CD28/CTLA4Ig fusion construct having a first amino acid sequence containing amino acid residues from about position 1 to about position 94 of the amino acid sequence corresponding to the extracellular domain of CD28, joined to a second amino acid sequence containing amino acid residues from about position 94 to about position 125 of the amino acid sequence corresponding to the extracellular domain of CTLA4, joined to a third amino acid sequence containing amino acids residues corresponding to the hinge, CH2 and CH3 regions of human IgCγ1. Other embodiments of the hybrid fusion proteins of the invention are described in Tables I and II and Example 7.

Also included in the invention is a method for regulating T cell interactions with other cells by inhibiting the interaction of CTLA4-positive T cells with B7 positive cells by reacting the T cells with ligands for the CTLA4 receptor. The ligands include B7Ig fusion protein, a monoclonal antibody reactive with CTLA4 receptor, and antibody fragments.

The invention also provides a method for regulating T cell interactions with B7 positive cells, using a ligand for the B7 antigen. Such a ligand is soluble CTLA4 fusion protein, e.g., CTLA4Ig fusion protein, of the invention, its fragments or derivatives, soluble CD28/CTLA4 hybrid fusion protein, e.g., the CD28/CTLA4Ig hybrid fusion protein, or a monoclonal antibody reactive with the B7 antigen.

The invention further includes a method for treating immune system diseases mediated by T cell interactions with B7 positive cells by administering a ligand reactive with B7 antigen to regulate T cell

interactions with B7 positive cells. The ligand is the CTLA4Ig fusion protein, or the CD28/CTLA4Ig fusion protein hybrid, or a monoclonal antibody reactive with B7 antigen.

A monoclonal antibody reactive with soluble CTLA4 fusion protein and a monoclonal antibody reactive with soluble CD28/CTLA4 fusion protein are described for use in regulating cellular interactions.

A novel Chinese Hamster Ovary cell line stably expressing the CTLA4Ig fusion protein is also disclosed.

Further, the present invention provides a method for blocking B7 interaction so as to regulate the immune response. This method comprises contacting lymphocytes with a B7-binding molecule and an IL4-binding molecule.

Additionally, the present invention provides a method for regulating an immune response which comprises contacting B7-positive lymphocytes with a B7-binding molecule and an IL4-binding molecule.

Also, the invention provides method for inhibiting tissue transplant rejection by a subject, the subject being a recipient of transplanted tissue. This method comprises administering to the subject a B7-binding molecule and an IL4-binding molecule.

The present invention further provides a method for inhibiting graft versus host disease in a subject which comprises administering to the subject a B7-binding molecule and an IL4-binding molecule.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a diagrammatic representation of CTLA4Ig fusion constructs as described in Example 2, infra.

Figure 2 is a photograph of a gel obtained from SDS-PAGE chromatographic purification of CTLA4Ig as described in Example 2, infra.

Figure 3 depicts the complete amino acid sequence encoding human CTLA4 receptor (SEQ ID NOs: 13 and 14) fused to the oncostatin M signal peptide (position -25 to -1), and including the newly identified N-linked glycosylation site (position 109-111), as described in Example 3, infra.

Figure 4 depicts the results of FACS^R analysis of binding of the B7Ig fusion protein to CD28- and CTLA4-transfected COS cells as described in Example 4, infra.

Figure 5 depicts the results of FACS^R analysis of binding of purified CTLA4Ig on B7 antigen-positive (B7$^+$) CHO cells and on a lymphoblastoid cell line (PM LCL) as described in Example 4, infra.

Figure 6 is a graph illustrating competition binding analysis of ^{125}I labeled B7Ig to immobilized CTLA4Ig as described in Example 4, infra.

Figure 7 is a graph showing the results of Scatchard analysis of ^{125}I-labeled B7Ig binding to immobilized CTLA4Ig as described in Example 4, infra.

Figure 8 is a photograph of a gel from SDS-PAGE chromatography of immunoprecipitation analysis of B7 positive CHO cells and PM LCL cells surface-labeled with ^{125}I as described in Example 4, infra.

Figure 9 is a graph depicting the effects on proliferation of T cells of CTLA4Ig as measured by [^{3}H]-thymidine incorporation as described in Example 4, infra.

Figure 10 is a bar graph illustrating the effects of CTLA4Ig on

helper T cell (T_h)-induced immunoglobulin secretion by human B cells as determined by enzyme immunoassay (ELISA) as described in Example 4, infra.

Figures 11A, 11B, and 11C are line graphs showing the survival of human pancreatic islet xenografts.

Figures 12A, 12B, 12C, and 12D are photographs of histopathology slides of human islets transplanted under the kidney capsule of B10 mice.

Figure 13 is a line graph showing the prolongation of islet graft survival with MAb to human B7.

Figure 14 is a line graph showing induction of donor-specific unresponsiveness to islet graft antigens by CTLA4Ig.

Figure 15 is a line graph showing antibody serum titer levels of mice injected with sheep red blood cells (SRBC), mAb L6 and rat Ig, mAb L6 and anti-IL4, CTLA4Ig and rat Ig, CTLA4Ig and anti-IL4. The X axis measures the antibody-serum titer. The Y axis measures time in days. The closed box represents mice injected with SRBC at day 0 and day 46. The open box represents mice injected with SRBC at day 46. The closed circle represents mice injected with mAb L6 and rat immunoglobulin. The open circle represents mice injected with mAb L6 and anti-IL4 antibody. The closed triangle represents mice injected with CTLA4Ig and rat immunoglobulin. The open triangle represents mice injected with CTLA4Ig and anti-IL4 antibody.

Figure 16 is a line graph showing antibody serum titer levels of mice injected with KLH, mAb L6 and rat Ig, mAb L6 and anti-IL4, CTLA4Ig and rat Ig, CTLA4Ig and anti-IL4. The X axis measures the antibody-serum titer. The Y axis measures time in days. The closed box represents mice injected with keyhole limpet hemocyanin (KLH) at day 46. The closed circle represents mice injected with

mAb L6 and rat immunoglobulin. The open circle represents mice injected with mAb L6 and anti-IL4 antibody. The closed triangle represents mice injected with CTLA4Ig and rat immunoglobulin. The open triangle represents mice injected with CTLA4Ig and anti-IL4 antibody.

Figure 17 is a graph showing the sequencing alignment of CD28 and CTLA4 family members. Sequences of human (H), mouse (M), rat (R), and chicken (Ch) CD28 are aligned with human and mouse CTLA4. Residues are numbered from the mature protein N-terminus with the signal peptides and transmembrane domains underlined and the CDR-analogous regions noted. Dark shaded areas highlight complete conservation of residues while light shaded areas highlight conservative amino acid substitutions in all family members.

Figure 18 is a line graph showing CTLA4Ig and CD28Ig mutants bind B7-1.

Figure 19 is a schematic map of CTLA4/CD28Ig hybrid fusion proteins. Open areas represent CD28 sequence; filled areas represent CTLA4 sequence; cross-hatched areas represent beginning of IgG Fc (also refer to Table I).

Figures 20A/B. A line graph showing that CTLA4/CD28Ig hybrid fusion proteins bind with high avidity to B7-1 CHO cells.

Figure 21. Molecular model of monomeric CTLA4Ig v-like extracellular domain.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITION

As used in this application, the following words or phrases have the meanings specified.

As used herein "blocking B7 interaction" means to interfere with the binding of the B7 antigen to its ligands such as CD28 and/or CTLA4 thereby obstructing T cell and B cell interaction.

As used herein a "B7-binding molecule" means any molecule which will bind the B7 antigen.

As used herein an "IL4-binding molecule" means any molecule which will recognize and bind to IL4.

As used herein a "CTLA4 mutant" means a molecule having amino acids which are similar to the amino acid sequence of the extracellular domain of CTLA4 so that the molecule recognizes and binds a B7 antigen.

As used herein a "CD28 mutant" means a molecule having amino acids which are similar to the amino acid sequence of the extracellular domain of CD28 so that the molecule recognizes and binds a B7 antigen.

As used herein a "CTLA4/CD28 hybrid fusion protein" is a molecule having at least portions of the extracellular domains of both CTLA4 and CD28 so that the molecule recognizes and binds a B7 antigen.

In order that the invention herein described may be more fully understood, the following description is set forth.

This invention is directed to the isolation and expression of the human CTLA4 receptor found on T cell surfaces, which binds to the B7 antigen expressed on activated B cells, and cells of other lineages, and to expression of soluble fusion protein products of the CTLA4 receptor gene. The invention also provides methods for using the expressed CTLA4 receptor to regulate cellular interactions, including T cell interactions with B7 positive cells.

In a preferred embodiment, the complete and correct DNA sequence encoding the amino acid sequence corresponding to human CTLA4 receptor protein of the invention is cloned using PCR. The cDNA containing the complete predicted coding sequence of CTLA4 was assembled from two PCR fragments amplified from H38 RNA, and inserted into the expression vector, CDM8 as described in detail in the Examples, infra. Isolates were transfected into COS cells and tested for binding of B7Ig, a soluble fusion protein having an amino acid sequence corresponding to the extracellular domain of B7 and a human immunoglobulin (Ig) Cγ1 region, as described by Linsley et al., J. Exp. Med. 173:721-730 (1991).

The DNA sequence of one isolate, designated as OMCTLA4, was then determined and found to correspond exactly to the predicted human CTLA4 sequence, fused at the N-terminus to the signal peptide from oncostatin M. The CTLA4 receptor is encoded by 187 amino acids (exclusive of the signal peptide and stop codons) and includes a newly identified N-linked glycosylation site at amino acid positions 109-111 (see Figure 3, infra). The CTLA4 receptor is expressed using the oncostatin M signal peptide.

In another preferred embodiment, soluble forms of the protein product of the CTLA4 receptor gene (CTLA4Ig) are prepared using fusion proteins having a first amino acid sequence corresponding to the extracellular domain of CTLA4 and a second amino acid sequence corresponding to the human IgCγ1 domain.

Cloning and expression plasmids (CDM8 and πLN) were constructed containing cDNAs encoding portions of the amino acid sequence corresponding to human CTLA4 receptor based on the cDNA sequence described herein, where the cDNA encoding a first amino acid sequence corresponding to a fragment of the extracellular domain of the CTLA4 receptor gene is joined to DNA encoding a second amino acid sequence corresponding to an IgC region that permits the expression of the CTLA4 receptor gene by altering the solubility of

the expressed CTLA4 protein.

Thus, soluble CTLA4Ig fusion protein is encoded by a first amino acid sequence containing amino acid residues from about position 1 to about position 125 of the amino acid sequence corresponding to the extracellular domain of CTLA4 joined to a second amino acid sequence containing amino acid residues corresponding to the hinge, CH2 and CH3 regions of human IgCγ1. The fusion protein is preferably produced in dimeric form. The construct was then transfected into COS or CHO cells, and CTLA4Ig was purified and identified as a dimer.

In accordance with the practice of this invention, CTLA4Ig and the CTL44/CD28 ~~CTL4/CD28~~ fusion protein hybrid may have amino acid substitutions in the amino acid sequence corresponding to the external domain of CTLA4 so as to produce molecules which would retain the functional property of CTLA4, namely, the molecule having such substitutions will still bind the B7 antigen. These amino acid substitutions include, but are not necessarily limited to, amino acid substitutions known in the art as "conservative".

For example, it is a well-established principle of protein chemistry that certain amino acid substitutions, entitled "conservative amino acid substitutions," can frequently be made in a protein without altering either the conformation or the function of the protein. Such changes include substituting any of isoleucine (I), valine (V), and leucine (L) for any other of these hydrophobic amino acids; aspartic acid (D) for glutamic acid (E) and vice versa; glutamine (Q) for asparagine (N) and vice versa; and serine (S) for threonine (T) and vice versa. Other substitutions can also be considered conservative, depending on the environment of the particular amino acid and its role in the three-dimensional structure of the protein. For example, glycine (G) and alanine (A) can frequently be interchangeable, as can alanine and valine (V).

Methionine (M), which is relatively hydrophobic, can frequently be interchanged with leucine and isoleucine, and sometimes with valine. Lysine (K) and arginine (R) are frequently interchangeable in locations in which the significant feature of the amino acid residue is its charge and the differing pK's of these two amino acid residues are not significant. Still other changes can be considered "conservative" in particular environments.

In fact, using the methodologies disclosed herein, mutants of the B7-binding molecule were produced. One mutant comprises (1) a sequence beginning with the amino acid at position 1 and ending with the amino acid at position 95 of the CD28 receptor protein; (2) a sequence beginning with the amino acid at position 95 and ending with amino acid at position 125 of the extracellular domain of CTLA4; and (3) a sequence corresponding to the human IgCγ1 domain.

The second mutant comprises (1) a sequence beginning with the amino acid at position 1 and ending with the amino acid at position 95 of the CD28 receptor protein; (2) a sequence beginning with the amino acid at position 95 and ending with amino acid at position 120 of the extracellular domain of CTLA4; and (3) a sequence corresponding to the human IgCγ1 domain.

The present invention provides a method for blocking B7 interaction so as to regulate the immune response which comprises contacting lymphocytes with a B7-binding molecule and an IL4-binding molecule. The lymphocytes may be B7 positive lymphocytes.

Further, the present invention provides a method for regulating an immune response which comprises contacting B7-positive lymphocytes with a B7-binding molecule and an IL4-binding molecule.

The immune response may be a B cell response resulting in the inhibition of antibody production. Additionally, the immune

response may be a T cell response resulting in inhibition of cell mediated immunity. Further, the immune response may be an inhibition of lymphocyte proliferation.

Also, the present invention provides a method for inhibiting tissue transplant rejection by a subject, the subject being a recipient of transplanted tissue. This method can comprise administering to the subject a B7-binding molecule and an IL4-binding molecule.

The invention further provides a method for inhibiting graft versus host disease in a subject which comprises administering to the subject a B7-binding molecule and an IL4-binding molecule.

In accordance with the practice of this invention, the B7-binding molecule may be a CTLA4Ig fusion protein. For example, the CTLA4Ig fusion protein may be a fusion protein having a first amino acid sequence containing amino acid residues from about position 1 to about position 125 of the amino acid sequence corresponding to the extracellular domain of CTLA4 and a second amino acid sequence containing amino acid residues corresponding to the hinge, CH2 and CH3 regions of human immunoglobulin Cγ1.

Alternatively, the B7-binding molecule may be a soluble CD28/CTLA4 hybrid fusion protein. For example, the CD28/CTLA4Ig fusion protein hybrid may be a fusion protein hybrid having a first amino acid sequence corresponding to a portion of the extracellular domain of CD28 receptor fused to a second amino acid sequence corresponding to a portion of the extracellular domain of CTLA4 receptor and a third amino acid sequence corresponding to the hinge, CH2 and CH3 regions of human immunoglobulin Cγ1.

Further, the IL4-binding molecule may be a monoclonal antibody which specifically recognizes and binds to IL4. Alternatively, the IL4-binding molecule is a soluble IL4 receptor which recognizes and binds to IL4 (Fanslow et al. 1991).

DNA encoding the amino acid sequence corresponding to the CTLA4Ig fusion protein has been deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland, under the provisions of the Budapest Treaty on May 31, 1991 and has been accorded ATCC accession number: 68629.

The present invention provides the first protein product of CTLA4 transcripts in the form of a soluble fusion protein. The CTLA4Ig protein forms a disulfide-linked dimer having two subunits, each of which has an M_r of approximately 50,000 indicating that native CTLA4 probably exists on the T cell surface as a disulfide-linked homodimer.

B7 antigen has been shown to be a ligand for CD28 receptor on T cells (Linsley et al., Proc. Natl. Acad. Sci. USA, supra). The CTLA4 receptor molecule appears functionally and structurally related to the CD28 receptor; both are receptors for the B cell activation antigen, B7, while CTLA4 appears to have higher affinity for B7, among the highest yet reported for lymphoid adhesion systems. However, CTLA4Ig was shown to bind more strongly to B7 positive ($B7^+$) cell lines than CD28Ig. Other experiments demonstrated that CTLA4 is a higher affinity receptor for B7 antigen than CD28 receptor. Additionally, CTLA4Ig was shown to bind a single protein on lymphoblastoid cells which is similar in size to the B7 antigen. CTLA4Ig inhibited T cell proliferation and inhibited T_h-induced IgM production.

In another preferred embodiment, hybrid fusion proteins having amino acid sequences corresponding to fragments of different receptor proteins were constructed. For example, amino acid sequences corresponding to selected fragments of the extracellular domains of CD28 and CTLA4 were linked to form soluble CD28/CTLA4 hybrid fusion proteins, e.g. a CD28/CTLA4Ig fusion protein. This protein was obtained having a first amino acid sequence containing amino acid residues corresponding to a fragment of the

extracellular domain of CD28 joined to a second amino acid sequence corresponding to a fragment of the extracellular domain of CTLA4Ig and to a third amino acid sequence corresponding to the hinge, CH2 and CH3 regions of human IgCγ1.

One embodiment of the hybrid fusion proteins is a CD28/CTLA4Ig fusion construct having a first amino acid sequence containing amino acid residues from about position 1 to about position 94 of the amino acid sequence corresponding to the extracellular domain of CD28, joined to a second amino acid sequence containing amino acid residues from about position 94 to about position 125 of the amino acid sequence corresponding to the extracellular domain of CTLA4, joined to a third amino acid sequence corresponding to the hinge, CH2 and CH3 regions of human IgCγ1.

The techniques for cloning and expressing DNA sequences encoding the amino acid sequences corresponding to the CTLA4 receptor protein, soluble fusion proteins and hybrid fusion proteins, e.g synthesis of oligonucleotides, PCR, transforming cells, constructing vectors, expression systems, and the like are well-established in the art, and most practitioners are familiar with the standard resource materials for specific conditions and procedures. However, the following paragraphs are provided for convenience and notation of modifications where necessary, and may serve as a guideline.

Cloning and Expression of Coding Sequences for Receptors and Fusion Proteins

Fusion protein constructs corresponding to CD28IgCγ1 and B7IgCγ1 for characterizing the CTLA4Ig of the present invention, and for preparing CD28/CTLA4 hybrid fusion proteins, were prepared as described by Linsley et al., J. Exp. Med. 173:721-730 (1991), incorporated by reference herein. Alternatively, cDNA clones may be prepared from RNA obtained from cells expressing B7 antigen and

CD28 receptor based on knowledge of the published sequences for these proteins (Aruffo and Seed, and Freeman, supra) using standard procedures.

INS D2

CTLA4Ig fusions consisting of DNA encoding amino acid sequences corresponding to the extracellular domain of CTLA4 and the hinge, CH2 and CH3 regions of human IgCγ1 were constructed by ligation of PCR fragments. The cDNA encoding the amino acid sequences is amplified using the polymerase chain reaction ("PCR") technique (U.S. Patent Nos. 4,683,195 and 4,683,202 to Mullis et al. and Mullis & Faloona, Methods Enzymol. 154:335-350 (1987)). CTLA4Ig fusion polypeptides were obtained having DNA encoding amino acid sequences containing amino acid residues from about position 1 to about position 125 of the amino acid sequence corresponding to the extracellular domain of CTLA4 and DNA encoding amino acid sequences corresponding to the hinge, CH2 and CH3 regions of Ig Cγ1.

INS D3

Because the expression of CTLA4 receptor protein in human lymphoid cells has not been previously reported, it was necessary to locate a source of CTLA4 mRNA. PCR cDNA made from the total cellular RNA of several human leukemia cell lines was screened, using as primers, oligonucleotides from the published sequence of the CTLA4 gene (Dariavach et al., supra). Of the cDNA tested, H38 cells (an HTLV II-associated leukemia line) provided the best yield of PCR products having the expected size. Since a signal peptide for CTLA4 was not identified in the CTLA4 gene, the N terminus of the predicted sequence of CTLA4 was fused to the signal peptide of oncostatin M (Malik et al., Molec. and Cell. Biol. 9:2847 (1989)) in two steps using oligonucleotides as described in the Examples, infra. The product of the PCR reaction was ligated with cDNA encoding the amino acid sequences corresponding to the hinge, CH2 and CH3 regions of Ig Cγ1 into a expression vector, such as CDM8 or πLN.

To obtain DNA encoding full length human CTLA4, a cDNA encoding the

transmembrane and cytoplasmic domains of CTLA4 was obtained by PCR from H38 cells and joined with a fragment from CTLA4Ig, obtained as described above, encoding the oncostatin M signal peptide fused to the N terminus of CTLA4, using oligonucleotide primers as described in the Examples, infra. PCR fragments were ligated into the plasmid CDM8, resulting in an expression plasmid encoding the full length CTLA4 gene, and designated OMCTLA4.

For construction of DNA encoding the amino acid sequence corresponding to hybrid fusion proteins, DNA encoding amino acids corresponding to portions of the extracellular domain of one receptor gene is joined to DNA encoding amino acids corresponding to portions of the extracellular domain of another receptor gene, and to DNA encoding the amino acid sequences corresponding to the hinge, CH2 and CH3 regions of human IgCγ1 using procedures as described above for the B7Ig, CD28Ig and CTLA4Ig constructs. Thus, for example, DNA encoding amino acid residues from about position 1 to about position 94 of the amino acid sequence corresponding to the extracellular domain of the CD28 receptor is joined to DNA encoding amino acid residues from about position 94 to about position 125 of the amino acid sequence corresponding to the extracellular domain of the CTLA4 receptor and to DNA encoding the amino acid sequences corresponding to the hinge, CH2 and CH3 regions of human IgCγ1.

To produce large quantities of cloned DNA, vectors containing DNA encoding the fusion constructs of the invention are transformed into suitable host cells, such as the bacterial cell line E. coli strain MC1061/p3 (Invitrogen Corp., San Diego, CA) using standard procedures, and colonies are screened for the appropriate plasmids.

The clones containing DNA encoding fusion constructs obtained as described above are then transfected into suitable host cells for expression. Depending on the host cell used, transfection is performed using standard techniques appropriate to such cells. For

example, transfection into mammalian cells is accomplished using DEAE-dextran mediated transfection, $CaPO_4$ co-precipitation, lipofection, electroporation, or protoplast fusion, and other methods known in the art including: lysozyme fusion or erythrocyte fusion, scraping, direct uptake, osmotic or sucrose shock, direct microinjection, indirect microinjection such as via erythrocyte-mediated techniques, and/or by subjecting host cells to electric currents. The above list of transfection techniques is not considered to be exhaustive, as other procedures for introducing genetic information into cells will no doubt be developed.

Expression in eukaryotic host cell cultures derived from multicellular organisms is preferred (Tissue Cultures, Academic Press, Cruz and Patterson, Eds. (1973)). These systems have the additional advantage of the ability to splice out introns and thus can be used directly to express genomic fragments. Useful host cell lines include Chinese hamster ovary (CHO), monkey kidney (COS), VERO and HeLa cells. In the present invention, cell lines stably expressing the fusion constructs are preferred.

Expression vectors for such cells ordinarily include promoters and control sequences compatible with mammalian cells such as, for example, CMV promoter (CDM8 vector) and avian sarcoma virus (ASV) (πLN vector). Other commonly used early and late promoters include those from Simian Virus 40 (SV 40) (Fiers, et al., Nature 273:113 (1973)), or other viral promoters such as those derived from polyoma, Adenovirus 2, and bovine papilloma virus. The controllable promoter, hMTII (Karin, et al., Nature 299:797-802 (1982)) may also be used. General aspects of mammalian cell host system transformations have been described by Axel (U.S. Patent No. 4,399,216 issued Aug. 16, 1983). It now appears, that "enhancer" regions are important in optimizing expression; these are, generally, sequences found upstream or downstream of the promoter region in non-coding DNA regions. Origins of replication may be obtained, if needed, from viral sources. However, integration into

the chromosome is a common mechanism for DNA replication in eukaryotes.

Although preferred host cells for expression of the fusion constructs include eukaryotic cells such as COS or CHO cells, other eukaryotic microbes may be used as hosts. Laboratory strains of Saccharomyces cerevisiae, Baker's yeast, are most used although other strains such as Schizosaccharomyces pombe may be used. Vectors employing, for example, the 2μ origin of replication of Broach, Meth. Enz. 101:307 (1983), or other yeast compatible origins of replications (for example, Stinchcomb et al., Nature 282:39 (1979)); Tschempe et al., Gene 10:157 (1980); and Clarke et al., Meth. Enz. 101:300 (1983)) may be used. Control sequences for yeast vectors include promoters for the synthesis of glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg. 7:149 (1968); Holland et al., Biochemistry 17:4900 (1978)). Additional promoters known in the art include the CMV promoter provided in the CDM8 vector (Toyama and Okayama, FEBS 268:217-221 (1990); the promoter for 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem. 255:2073 (1980)), and those for other glycolytic enzymes. Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and enzymes responsible for maltose and galactose utilization. It is also believed terminator sequences are desirable at the 3' end of the coding sequences. Such terminators are found in the 3' untranslated region following the coding sequences in yeast-derived genes.

Alternatively, prokaryotic cells may be used as hosts for expression. Prokaryotes most frequently are represented by various strains of E. coli; however, other microbial strains may also be used. Commonly used prokaryotic control sequences which are defined herein to include promoters for transcription initiation,

optionally with an operator, along with ribosome binding site sequences, include such commonly used promoters as the beta-lactamase (penicillinase) and lactose (lac) promoter systems (Chang et al., Nature 198: 1056 (1977)), the tryptophan (trp) promoter system (Goeddel et al., Nucleic Acids Res. 8:4057 (1980)) and the lambda derived P_L promoter and N-gene ribosome binding site (Shimatake et al., Nature 292:128 (1981)).

The nucleotide sequences encoding CD28Ig and CTLA4Ig proteins, and fusion hybrid proteins such as CD28/CTLA4Ig, may be expressed in a variety of systems as set forth below. The cDNA may be excised by suitable restriction enzymes and ligated into suitable prokaryotic or eukaryotic expression vectors for such expression. Because CD28 and CTLA4 receptor proteins occur in nature as dimers, it is believed that successful expression of these proteins requires an expression system which permits these proteins to form as dimers. Truncated versions of these proteins (i.e. formed by introduction of a stop codon into the sequence at a position upstream of the transmembrane region of the protein) appear not to be expressed. The expression of CD28 and CTLA4 receptors as fusion proteins permits dimer formation of these proteins. Thus, expression of CTLA4 protein as a fusion product is preferred in the present invention.

A stable CHO line of the invention, designated Chinese Hamster Ovary Cell Line CTLA4Ig-24, is preferred for expression of CTLA4Ig and has been deposited with the ATCC under the terms of the Budapest Treaty on May 31, 1991, and accorded ATCC accession number 10762.

Expression of the CTLA4 receptor of the invention is accomplished transfecting a cell line such as COS cells, and detecting expression by binding of the CTLA4-transfected cells to a ligand for the CTLA4 receptor, for example by testing for binding of the cells to B7Ig fusion protein.

Sequences of the resulting constructs are confirmed by DNA sequencing using known procedures, for example as described by Sanger et al., Proc. Natl. Acad. Sci. USA 74:5463 (1977), as further described by Messing et al., Nucleic Acids Res. 9:309 (1981), or by the method of Maxam et al. Methods Enzymol. 65:499 (1980)).

Recovery of Protein Products

As noted above, CD28 and CTLA4 receptor genes are not readily expressed as mature proteins using direct expression of DNA encoding the truncated protein. To enable homodimer formation, DNA encoding the amino acid sequence corresponding to the extracellular domains of CD28 and CTLA4, and including the codons for a signal sequence such as that of oncostatin M in cells capable of appropriate processing, is fused with DNA encoding the amino acid sequence corresponding to the Fc domain of a naturally dimeric protein. Purification of these fusion protein products after secretion from the cells is thus facilitated using antibodies reactive with the anti-immunoglobulin portion of the fusion proteins. When secreted into the medium, the fusion protein product is recovered using standard protein purification techniques, for example by application to protein A columns.

USE

CTLA4Ig fusion protein and/or fragments of the fusion protein may be used to react with B7 positive cells, such as B cells, to regulate immune responses mediated by T cell interactions with the B7 antigen positive cells or in vitro for leukocyte typing so as to define B cell maturational stages and/or B cell associated diseases (Yokochi et al. J. Immuno. 128(2):823. Surface immunostaining of leukocytes is accomplished by immunofluorescent technology or immunoenzymatic methods but other means of detection are possible.

Soluble CTLA4 proteins and CTLA4/CD28 hybrid fusion proteins, and/or fragments and derivatives of these proteins, may also be used to react with B7 positive cells, including B cells, to regulate immune responses mediated by T cell dependent B cell responses. The term "fragment" as used herein means a portion of the amino acid sequence encoding the protein referred to as "CTLA4". A fragment of the soluble CTLA4 protein that may be used is a polypeptide having an amino acid sequence corresponding to some portion of the amino acid sequence corresponding to the CTLA4 receptor used to obtain the soluble CTLA4 protein as described herein.

The B7 antigen expressed on activated B cells and cells of other lineages, and the CD28 receptor expressed on T cells, can directly bind to each other, and this interaction can mediate cell-cell interaction. Such interactions directly trigger the CD28 activation pathway in T cells, leading to cytokine production, T cell proliferation, and B cell differentiation into immunoglobulin producing cells. The activation of B cells that occurs, can cause increased expression of B7 antigen and further CD28 stimulation, leading to a state of chronic inflammation such as in autoimmune diseases, allograft rejection, graft versus host disease or chronic allergic reactions. Blocking or inhibiting this reaction may be effective in preventing T cell cytokine production and thus preventing or reversing inflammatory reactions.

Soluble CTLA4, e.g. CTLA4Ig, is shown herein to be a potent inhibitor of <u>in vitro</u> lymphocyte functions requiring T and B cell interaction. This indicates the importance of interactions between the B7 antigen and its counter-receptors, CTLA4 and/or CD28. The cytoplasmic domains of murine and human CTLA4 are similar (Dariavach et al., <u>supra</u>, 1988), suggesting that this region has important functional properties. The cytoplasmic domains of CD28 and CTLA4 also share homology.

CTLA4 is a more potent inhibitor in vitro of lymphocyte responses than either anti-BB1, or anti-CD28 mAbs. CTLA4Ig does not have direct stimulatory effects on T cell proliferation to counteract its inhibitory effects. Therefore, the CTLA4Ig fusion protein may perform as a better inhibitor in vivo than anti-CD28 monoclonal antibodies. The immunosuppressive effects of CTLA4Ig in vitro suggests its use in therapy for treatment of autoimmune disorders involving abnormal T cell activation or Ig production.

The CTLA4Ig fusion protein is expected to exhibit inhibitory properties in vivo. Thus, it is expected that CTLA4Ig will act to inhibit T cells in a manner similar to the effects observed for the anti-CD28 antibody, under similar conditions in vivo. Under conditions where T cell/B cell interactions are occurring as a result of contact between T cells and B cells, binding of introduced CTLA4Ig to react with B7 antigen positive cells, for example B cells, may interfere, i.e. inhibit, the T cell/B cell interactions resulting in regulation of immune responses. Because of this exclusively inhibitory effect, CTLA4Ig is expected to be useful in vivo as an inhibitor of T cell activity, over non-specific inhibitors such as cyclosporine and glucosteroids.

In one embodiment, the CTLA4Ig fusion protein or CTLA4/CD28Ig hybrid proteins, may be introduced in a suitable pharmaceutical carrier in vivo, i.e. administered into a human subject for treatment of pathological conditions such as immune system diseases or cancer.

Introduction of the fusion protein in vivo is expected to result in interference with T cell interactions with other cells, such as B cells, as a result of binding of the ligand to B7 positive cells. The prevention of normal T cell interactions may result in decreased T cell activity, for example, decreased T cell proliferation. In addition, administration of the fusion protein in vivo is expected to result in regulation of in vivo levels of

cytokines, including, but not limited to, interleukins, e.g. interleukin ("IL")-2, IL-3, IL-4, IL-6, IL-8, growth factors including tumor growth factor ("TGF"), colony stimulating factor ("CSF"), interferons ("IFNs"), and tumor necrosis factor ("TNF") to promote desired effects in a subject. For example, when the fusion protein is introduced in vivo, it may block production of cytokines, which contribute to malignant growth, for example of tumor cells. The fusion protein may also block proliferation of viruses dependent on T cell activation, such as the virus that causes AIDS, HTLV1.

Under some circumstances, as noted above, the effect of administration of the CTLA4Ig fusion protein or its fragments in vivo is inhibitory, resulting from blocking by the fusion protein of the CTLA4 and CD28 triggering resulting from T cell/B cell contact. For example, the CTLA4Ig protein may block T cell proliferation. Introduction of the CTLA4Ig fusion protein in vivo will thus produce effects on both T and B cell-mediated immune responses. The fusion protein may also be administered to a subject in combination with the introduction of cytokines or other therapeutic reagents.

In an additional embodiment of the invention, other reagents, including derivatives reactive with the CTLA4Ig fusion protein or the CTLA4 receptor are used to regulate T cell interactions. For example, antibodies, and/or antibody fragments reactive with the CTLA4 receptor may be screened to identify those capable of inhibiting the binding of the CTLA4Ig fusion protein to the B7 antigen. The antibodies or antibody fragments such as Fab or $F(ab')_2$ fragments, may then be used to react with the T cells, for example, to inhibit T cell proliferation.

Monoclonal antibodies reactive with CTLA4 receptor, may be produced by hybridomas prepared using known procedures, such as those introduced by Kohler and Milstein (Kohler and Milstein, Nature,

256:495-97 (1975)), and modifications thereof, to regulate cellular interactions.

These techniques involve the use of an animal which is primed to produce a particular antibody. The animal can be primed by injection of an immunogen (e.g. the B7Ig fusion protein, CTLA4Ig fusion protein or CD28/CTLA4Ig hybrid fusion protein or other functional, soluble forms thereof) to elicit the desired immune response, i.e. production of antibodies from the primed animal. A primed animal is also one which is expressing a disease. Lymphocytes derived from the lymph nodes, spleens or peripheral blood of primed, diseased animals can be used to search for a particular antibody. The lymphocyte chromosomes encoding desired immunoglobulins are immortalized by fusing the lymphocytes with myeloma cells, generally in the presence of a fusing agent such as polyethylene glycol (PEG). Any of a number of myeloma cell lines may be used as a fusion partner according to standard techniques; for example, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653, Sp2/0-Ag14, or HL1-653 myeloma lines. These myeloma lines are available from the ATCC, Rockville, Maryland.

The resulting cells, which include the desired hybridomas, are then grown in a selective medium such as HAT medium, in which unfused parental myeloma or lymphocyte cells eventually die. Only the hybridoma cells survive and can be grown under limiting dilution conditions to obtain isolated clones. The supernatants of the hybridomas are screened for the presence of the desired specificity, e.g. by immunoassay techniques using the CTLA4Ig protein that has been used for immunization. Positive clones can then be subcloned under limiting dilution conditions, and the monoclonal antibody produced can be isolated.

Various conventional methods can be used for isolation and purification of the monoclonal antibodies so as to obtain them free from other proteins and contaminants. Commonly used methods for

purifying monoclonal antibodies include ammonium sulfate precipitation, ion exchange chromatography, and affinity chromatography (Zola et al., in Monoclonal Hybridoma Antibodies: Techniques and Applications, Hurell (ed.) pp. 51-52 (CRC Press, 1982)). Hybridomas produced according to these methods can be propagated in vitro or in vivo (in ascites fluid) using techniques known in the art (Fink et al., Prog. Clin. Pathol., 9:121-33 (1984), Fig. 6-1 at p. 123).

Generally, the individual cell line may be propagated in vitro, for example, in laboratory culture vessels, and the culture medium containing high concentrations of a single specific monoclonal antibody can be harvested by decantation, filtration, or centrifugation.

In addition, fragments of these antibodies containing the active binding region reactive with the extracellular domain of CTLA4 receptor, such as Fab, $F(ab')_2$ and Fv fragments may be produced. Such fragments can be produced using techniques well established in the art (e.g. Rousseaux et al., in Methods Enzymol., 121:663-69, Academic Press (1986)).

Anti-B7 monoclonal antibodies prepared as described above may be used to bind to B7 antigen to inhibit interactions of CD28-positive or CTLA4-positive T cells with B7 positive cells. Anti-CTLA4 monoclonal antibodies may be used to bind to CTLA4 receptor to inhibit the interaction of CTLA4-positive T cells with other cells.

In another embodiment, the CTLA4Ig fusion protein may be used to identify additional compounds capable of regulating the interaction between CTLA4 and the B7 antigen. Such compounds may include small naturally occurring molecules that can be used to react with B cells and/or T cells. For example, fermentation broths may be tested for the ability to inhibit CTLA4/B7 interactions. In addition, derivatives of the CTLA4Ig fusion protein as described

above may be used to regulate T cell proliferation. For example, the fragments or derivatives may be used to block T cell proliferation in graft versus host (GVH) disease which accompanies allogeneic bone marrow transplantation.

The CD28-mediated T cell proliferation pathway is cyclosporine-resistant, in contrast to proliferation driven by the CD3/Ti cell receptor complex (June et al., 1987, supra). Cyclosporine is relatively ineffective as a treatment for GVH disease (Storb, Blood 68:119-125 (1986)). GVH disease is thought to be mediated by T lymphocytes which express CD28 antigen (Storb and Thomas, Immunol. Rev. 88:215-238 (1985)). Thus, the CTLA4Ig fusion protein may be useful alone, or in combination with immunosuppressants such as cyclosporine, for blocking T cell proliferation in GVH disease.

Regulation of CTLA4-positive T cell interactions with B7 positive cells, including B cells, by the methods of the invention may thus be used to treat pathological conditions such as autoimmunity, transplantation, infectious diseases and neoplasia.

The B7-binding molecules and IL4-binding molecules described herein may be in a variety of dosage forms which include, but are not limited to, liquid solutions or suspensions, tablets, pills, powders, suppositories, polymeric microcapsules or microvesicles, liposomes, and injectable or infusible solutions. The preferred form depends upon the mode of administration and the therapeutic application.

The most effective mode of administration and dosage regimen for the molecules of the present invention depends upon the severity and course of the disease, the subject's health and response to treatment and the judgment of the treating physician. Accordingly, the dosages of the molecules should be titrated to the individual subject.

The interrelationship of dosages for animals of various sizes and species and humans based on mg/m^2 of surface area is described by Freireich, E.J., et al. (Quantitative Comparison of Toxicity of Anticancer Agents in Mouse, Rat, Hamster, Dog, Monkey and Man. Cancer Chemother, Rep., 50, No.4, 219-244, May 1966).

Adjustments in the dosage regimen may be made to optimize the growth inhibiting response. Doses may be divided and administered on a daily basis or the dose may be reduced proportionally depending upon the situation. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the specific therapeutic situation.

In accordance with the practice of the invention an effective amount for treating a subject may be between about 0.1 and about 10mg/kg body weight of subject. Also, the effective amount may be an amount between about 1 and about 10 mg/kg body weight of subject.

Advantages of the Invention: The subject invention overcomes the problems associated with current therapies directed to preventing the rejection of tissue or organ transplants. In contrast to present therapies, the present invention affects only immunological responses mediated by B7 interactions.

For example, the present invention affects the transplant antigen-specific T cells, thus inducing donor-specific and antigen-specific tolerance. The binding of CD28 by its ligand, B7/BB1 (B7), during T cell receptor engagement is critical for proper T cell signaling in some systems (M. K. Jenkins, P. S. Taylor, S. D. Norton, K. B. Urdahl, J. Immunol. 147:2461 (1991); C. H. June, J. A. Ledbetter, P. S. Linsley, C. B. Thompson,Immunol. Today 11:211 (1990); H. Reiser, G. J. Freeman, Z. Razi-Wolf, C. D. Gimmi, B. Benacerraf, L. M. Nadler, Proc. Natl. Acad. Sci. U.S.A. 89:271 (1992); N. K. Damie, K. Klussman, P. S. Linsley, A. Aruffo, J.Immunol. 148:1985

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When the interaction of CD28 with its ligand is blocked, antigen-specific T cells are inappropriately induced into a state of antigen-specific T cell anergy (M. K. Jenkins, P. S. Taylor, S. D. Norton, K. B. Urdahl, J. Immunol. 147:2461 (1991); F. A. Harding, J. G. McArthur, J. A. Gross, D. H. Raulet, J. P. Allison, Nature 356:607 (1992)).

CTLA4Ig fusion protein binds to both human and murine B7 (with a 20-fold greater affinity than CD28), blocks the binding of CD28 to B7, inhibits T cell activation, and induces T cell unresponsiveness in vitro (F. A. Harding, J. G. McArthur, J. A. Gross, D. H. Raulet, J. P. Allison, Nature 356:607 (1992); P. S. Linsley et al., J. Exp. Med. 174:561 (1991).

Moreover, the present invention would be useful to obtain expression of a soluble protein product of the heretofore unexpressed CTLA4 gene, and to identify a natural ligand for CTLA4 that is involved in functional responses of T cells. The soluble protein product could then be used to regulate T cell responses in vivo to treat pathological conditions.

The following examples are presented to illustrate the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any way to otherwise limit the scope of the invention.

EXAMPLE 1

Preparation of B7Ig and CD28Ig Fusion Proteins

Receptor-immunoglobulin C gamma (IgCγ) fusion proteins B7Ig and CD28Ig were prepared as described by Linsley et al., in J. Exp. Med. 173:721-730 (1991), incorporated by reference herein.

Briefly, DNA encoding amino acid sequences corresponding to the respective receptor protein (e.g. B7) was joined to DNA encoding amino acid sequences corresponding to the hinge, CH2 and CH3 regions of human IgCγ1. This was accomplished as follows.

Polymerase Chain Reaction (PCR). For PCR, DNA fragments were amplified using primer pairs as described below for each fusion protein. PCR reactions (0.1 ml final volume) were run in Taq polymerase buffer (Stratagene, La Jolla, CA), containing 20 μmoles each of dNTP; 50-100 pmoles of the indicated primers; template (1 ng plasmid or cDNA synthesized from ≤ 1 μg total RNA using random hexamer primer, as described by Kawasaki in PCR Protocols, Academic Press, pp. 21-27 (1990), incorporated by reference herein); and Taq polymerase (Stratagene). Reactions were run on a thermocycler (Perkin Elmer Corp., Norwalk, CT) for 16-30 cycles (a typical cycle consisted of steps of 1 min at 94°C, 1-2 min at 50°C and 1-3 min at 72°C).

Plasmid Construction. Expression plasmids containing cDNA encoding CD28, as described by Aruffo and Seed, Proc. Natl. Acad. Sci. USA 84:8573 (1987)), were provided by Drs. Aruffo and Seed (Mass General Hospital, Boston, MA). Plasmids containing cDNA encoding CD5, as described by Aruffo, Cell 61:1303 (1990)), were provided by Dr. Aruffo. Plasmids containing cDNA encoding B7, as described by Freeman et al., J. Immunol. 143:2714 (1989)), were provided by Dr. Freeman (Dana Farber Cancer Institute, Boston, MA). For initial attempts at expression of soluble forms of CD28 and B7, constructs were made (OMCD28 and OMB7) as described by Linsley et al., J. Exp. Med., supra, in which stop codons were introduced upstream of the transmembrane domains and the native signal peptides were replaced with the signal peptide from oncostatin M (Malik et al., Mol. Cell Biol. 9:2847 (1989)). These were made using synthetic oligonucleotides for reconstruction (OMCD28) or as primers (OMB7) for PCR. OMCD28, is a CD28 cDNA modified for more efficient expression by replacing the signal peptide with the analogous

region from oncostatin M. CD28Ig and B7Ig fusion constructs were made in two parts. The 5' portions were made using OMCD28 and OMB7 as templates and the oligonucleotide, CTAGCCACTGAAGCTTCACCATGGGTGTACTGCTCACAC (SEQ ID NO:1), (encoding the amino acid sequence corresponding to the oncostatin M signal peptide) as a forward primer, and either TGGCATGGGCTCCTGATCAGGCTTAGAAGGTCCGGGAAA (SEQ ID NO:2), or, TTTGGGCTCCTGATCAGGAAAATGCTCTTGCTTGGTTGT (SEQ ID NO:3) as reverse primers, respectively. Products of the PCR reactions were cleaved with restriction endonucleases (Hind III and BclI) as sites introduced in the PCR primers and gel purified.

The 3' portion of the fusion constructs corresponding to human IgCγ1 sequences was made by a coupled reverse transcriptase (from Avian myeloblastosis virus; Life Sciences Associates, Bayport, NY)-PCR reaction using RNA from a myeloma cell line producing human-mouse chimeric mAb L6 (provided by Dr. P. Fell and M. Gayle, Bristol-Myers Squibb Company, Pharmaceutical Research Institute, Seattle, WA) as template. The oligonucleotide, AAGCAAGAGCATTTTCCTGATCAGGAGCCCAAATCTTCTGACAAAACTCACACATCCCCACCGTC CCCAGCACCTGAACTCCTG (SEQ ID NO:4), was used as forward primer, and CTTCGACCAGTCTAGAAGCATCCTCGTGCGACCGCGAGAGC (SEQ ID NO:5) as reverse primer. Reaction products were cleaved with BclI and XbaI and gel purified. Final constructs were assembled by ligating HindIII/BclI cleaved fragments containing CD28 or B7 sequences together with BclI/XbaI cleaved fragment containing IgCγ1 sequences into HindIII/XbaI cleaved CDM8. Ligation products were transformed into MC1061/p3 E. coli cells and colonies were screened for the appropriate plasmids. Sequences of the resulting constructs were confirmed by DNA sequencing.

The construct encoding B7 contained DNA encoding amino acids corresponding to amino acid residues from approximately position 1 to approximately position 215 of the extracellular domain of B7. The construct encoding CD28 contained DNA encoding amino acids

corresponding to amino acid residues from approximately position 1 to approximately position 134 of the extracellular domain of CD28.

CD5Ig was constructed in identical fashion, using CATTGCACAGTCAAGCTTCCATGCCCATGGGTTCTCTGGCCACCTTG (SEQ ID NO:6), as forward primer and ATCCACAGTGCAGTGATCATTTGGATCCTGGCATGTGAC (SEQ ID NO:7) as reverse primer. The PCR product was restriction endonuclease digested and ligated with the IgCγ1 fragment as described above. The resulting construct (CD5Ig) encoded a mature protein having an amino acid sequence containing amino acid residues from position 1 to position 347 of the sequence corresponding to CD5, two amino acids introduced by the construction procedure (amino acids DQ), followed by DNA encoding amino acids corresponding to the IgCγ1 hinge region.

INS D6

Cell Culture and Transfections. COS (monkey kidney cells) were transfected with expression plasmids expressing CD28 and B7 using a modification of the protocol of Seed and Aruffo (Proc. Natl. Acad. Sci. 84:3365 (1987)), incorporated by reference herein. Cells were seeded at 10^6 per 10 cm diameter culture dish 18-24 h before transfection. Plasmid DNA was added (approximately 15 μg/dish) in a volume of 5 mls of serum-free DMEM containing 0.1 mM chloroquine and 600 μg/ml DEAE Dextran, and cells were incubated for 3-3.5 h at 37°C. Transfected cells were then briefly treated (approximately 2 min) with 10% dimethyl sulfoxide in PBS and incubated at 37°C for 16-24 h in DMEM containing 10% FCS. At 24 h after transfection, culture medium was removed and replaced with serum-free DMEM (6 ml/dish). Incubation was continued for 3 days at 37°C, at which time the spent medium was collected and fresh serum-free medium was added. After an additional 3 days at 37°C, the spent medium was again collected and cells were discarded.

INS D7

CHO cells expressing CD28, CD5 or B7 were isolated as described by Linsley et al., (1991) supra, as follows: Briefly, stable transfectants expressing CD28, CD5, or B7, were isolated following

cotransfection of dihydrofolate reductase-deficient Chinese hamster ovary (dhfr$^-$ CHO) cells with a mixture of the appropriate expression plasmid and the selectable marker, pSV2dhfr (Linsley et al., Proc. Natl. Acad. Sci. USA 87:5031 (1990)), incorporated by reference herein. Transfectants were then grown in increasing concentrations of methotrexate to a final level of 1μM and were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 0.2 mM proline and 1 μM methotrexate. CHO lines expressing high levels of CD28 (CD28$^+$ CHO) or B7 (B7$^+$ CHO) were isolated by multiple rounds of fluorescence-activated cell sorting (FACS^R) following indirect immunostaining with mAbs 9.3 or BB-1. Amplified CHO cells negative for surface expression of CD28 or B7 (dhfr$^+$ CHO) were also isolated by FACS^R from CD28-transfected populations.

Immunostaining and FACS^R Analysis. Transfected CHO or COS cells or activated T cells were analyzed by indirect immunostaining. Before staining, CHO cells were removed from their culture vessels by incubation in PBS containing 10 mM EDTA. Cells were first incubated with murine mAbs 9.3 (Hansen et al., Immunogenetics 10:247 (1980)) or BB-1 (Yokochi et al., J. Immunol. 128:823 (1981)), or with Ig fusion proteins (all at 10 μg/ml in DMEM containing 10% FCS) for 1-2 h at 4°C. Cells were then washed, and incubated for an additional 0.5-2h at 4°C with a FITC-conjugated second step reagent (goat anti-mouse Ig serum for murine mAbs, or goat anti-human Ig Cγ serum for fusion proteins (Tago, Inc., Burlingame, CA)). Fluorescence was analyzed on a FACS IV^R cell sorter (Becton Dickinson and CO., Mountain View, CA) equipped with a four decade logarithmic amplifier.

Purification of Ig Fusion Proteins. The first, second and third collections of spent serum-free culture media from transfected COS cells were used as sources for the purification of Ig fusion proteins. After removal of cellular debris by low speed centrifugation, medium was applied to a column (approximately 200-400 ml medium/ml packed bed volume) of immobilized protein A

(Repligen Corp., Cambridge, MA) equilibrated with 0.05 M sodium citrate, pH 8.0. After application of the medium, the column was washed with 1 M potassium phosphate, pH 8, and bound protein was eluted with 0.05 M sodium citrate, pH 3. Fractions were collected and immediately neutralized by addition of 1/10 volume of 2 M Tris, pH 8. Fractions containing the peak of A_{280} absorbing material were pooled and dialyzed against PBS before use. Extinction coefficients of 2.4 and 2.8 ml/mg for CD28Ig and B7Ig, respectively, were determined by amino acid analysis of solutions of known absorbance. The recovery of purified CD28Ig and B7Ig binding activities was nearly quantitative as judged by FACS^R analysis after indirect fluorescent staining of $B7^+$ and $CD28^+$ CHO cells.

EXAMPLE 2

Preparation of CTLA4Ig Fusion Protein

A soluble genetic fusion encoding CTLA4Ig between the extracellular domain of CTLA4 and an IgCγ1 domain was constructed in a manner similar to that described above for the CD28Ig construct. The extracellular domain of the CTLA4 gene was cloned by PCR using synthetic oligonucleotides corresponding to the published sequence (Dariavach et al., Eur. Journ. Immunol. 18:1901-1905 (1988)).

Because a signal peptide for CTLA4 was not identified in the CTLA4 gene, the N-terminus of the predicted sequence of CTLA4 was fused to the signal peptide of oncostatin M (Malik et al., Mol. and Cell. Biol. 9:2847 (1989)) in two steps using overlapping oligonucleotides. For the first step, the oligonucleotide, CTCAGTCTGGTCCTTGCACTCCTGTTTCCAAGCATGGCGAGCATGGCAATGCACGTGGCCCAGCC (SEQ ID NO:8) (which encoded the C terminal 15 amino acids from the oncostatin M signal peptide fused to the N terminal 7 amino acids of CTLA4) was used as forward primer, and TTTGGGCTCCTGATCAGAATCTGGGCACGGTTG (SEQ ID NO:9) (encoding amino

acid residues 119-125 of the amino acid sequence encoding CTLA4 receptor and containing a Bcl I restriction enzyme site) as reverse primer. The template for this step was cDNA synthesized from 1 μg of total RNA from H38 cells (an HTLV II infected T cell leukemic cell line provided by Drs. Salahudin and Gallo, NCI, Bethesda, MD). A portion of the PCR product from the first step was reamplified, using an overlapping forward primer, encoding the N terminal portion of the oncostatin M signal peptide and containing a Hind III restriction endonuclease site, CTAGCCACTGAAGCTTCACCAATGGGTGTACTGCTCACACAGAGGACGCTGCTCAGTCTGGTCCT TGCACTC (SEQ ID NO:10) and the same reverse primer. The product of the PCR reaction was digested with Hind III and Bcl I and ligated together with a Bcl 1/Xba I cleaved cDNA fragment encoding the amino acid sequences corresponding to the hinge, CH2 and CH3 regions of IgCγ1 into the Hind III/Xba I cleaved expression vector, CDM8 or Hind III/Xba I cleaved expression vector πLN (provided by Dr. Aruffo).

A map of the resulting CTLA4Ig fusion construct is shown in Figure 1. Sequences displayed in this figure show the junctions between CTLA4 (upper case letters, unshaded regions) and the signal peptide, SP, of oncostatin M (dark shaded regions), and the hinge, H, of IgCγ1 (stippled regions). The amino acid in parentheses was introduced during construction. Asterisks (*) indicate cysteine to serine mutations introduced in the IgCγ hinge region. The immunoglobulin superfamily V-like domain present in CTLA4 is indicated, as are the CH2 and CH3 domains of IgCγ1.

Expression plasmids, CDM8, containing CTLA4Ig were then transfected into COS cells using DEAE/dextran transfection by modification (Linsley et al., 1991, supra) of the protocol described by Seed and Aruffo, 1987, supra.

Expression plasmid constructs (πLN or CDM8) containing cDNA encoding the amino acid sequence of CTLA4Ig, was transfected by

lipofection using standard procedures into dhfr$^-$ CHO lines to obtain novel cell lines stably expressing CTLA4Ig.

DNA encoding the amino acid sequence corresponding to CTLA4Ig has been deposited with the ATCC under the Budapest Treaty on May 31, 1991, and has been accorded ATCC accession number 68629.

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A preferred stable transfectant, expressing CTLA4Ig, designated Chinese Hamster Ovary Cell Line, CTLA4Ig-24, was made by screening B7 positive CHO cell lines for B7 binding activity in the medium using immunostaining. Transfectants were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 0.2 mM proline and 1 μM methotrexate.

The CTLA4Ig-24 CHO cell line has been deposited with the ATCC under the Budapest Treaty on May 31, 1991 and has been accorded accession number ATCC 10762.

CTLA4Ig was purified by protein A chromatography from serum-free conditioned supernatants (Figure 2). Concentrations of CTLA4Ig were determined assuming an extinction coefficient at 280 nm of 1.6 (experimentally determined by amino acid analysis of a solution of known absorbance). Molecular weight standards (lanes 1 and 3, Figure 2) and samples (1 μg) of CTLA4Ig (lanes 2 and 4) were subjected to SDS-PAGE (4-12% acrylamide gradient) under non-reducing conditions (-βME, lanes 1 and 2) or reducing conditions (+ βME, lanes 3 and 4) Proteins were visualized by staining with Coomassie Brilliant Blue.

Under non-reducing conditions, CTLA4Ig migrated as a M_r approximately 100,000 species, and under reducing conditions, as a M_r approximately 50,000 species (Figure 2). Because the IgC γ hinge disulfides were eliminated during construction, CTLA4Ig, like CD28Ig, is a dimer presumably joined through a native disulfide linkage.

EXAMPLE 3

CTLA4 Receptor

To reconstruct DNA encoding the amino acid sequence corresponding to the full length human CTLA4 gene, cDNA encoding amino acids corresponding to a fragment of the transmembrane and cytoplasmic domains of CTLA4 was cloned by PCR and then joined with cDNA encoding amino acids corresponding to a fragment from CTLA4Ig that corresponded to the oncostatin M signal peptide fused to the N-terminus of CTLA4. Procedures for PCR, and cell culture and transfections were as described above in Example 1 using COS cells and DEAE-dextran transfection.

Because the expression of CTLA4 receptor protein in human lymphoid cells has not been previously reported, it was necessary to locate a source of CTLA4 mRNA. PCR cDNA reverse transcribed from the total cellular RNA of H38 cells, as noted above, was used for cloning by PCR. For this purpose, the oligonucleotide,

GCAATGCACGTGGCCCAGCCTGCTGTGGTAGTG

(SEQ ID NO:11), (encoding the first 11 amino acids in the predicted coding sequence) was used as a forward primer, and

TGATGTAACATGTCTAGATCAATTGATGGGAATAAAATAAGGCTG

(SEQ ID NO:12) (homologous to the last 8 amino acids in CTLA4 and containing a Xba I site) as reverse primer. The template again was a cDNA synthesized from 1 µg RNA from H38 cells. Products of the PCR reaction were cleaved with the restriction endonucleases Nco I and Xba I and the resulting 316 bp product was gel purified. A 340 bp Hind III/Nco I fragment from the CTLAIg fusion described above was also gel-purified, and both restriction fragments were ligated into Hind III/Xba I cleaved CDM8 to form OMCTLA.

The resulting construct corresponded to full length CTLA4 (SEQ ID NOs: 13 and 14) and the oncostatin M signal peptide. The construct is shown in Figure 3 and was designated OMCTLA4. The sequence for

CTLA4 shown in Figure 3 differs from the predicted human CTLA4 DNA sequence (Dariavach et al., supra) by a base change such that the previously reported alanine at amino acid position 111 of the amino acid sequence shown, encodes a threonine. This threonine is part of a newly identified N-linked glycosylation site that may be important for successful expression of the fusion protein.

Ligation products were transformed into MC1061/p3 E. coli cells and colonies were screened for the appropriate plasmids. Sequences of the resulting constructs were confirmed by DNA sequence analysis.

EXAMPLE 4

Characterization of CTLA4Ig

To characterize the CTLA4Ig constructs, several isolates, CD28Ig, B7Ig, and CD5Ig, were prepared as described above and were transfected into COS cells as described in Examples 2 and 3, and were tested by FACS^R analysis for binding of B7Ig. In addition to the above-mentioned constructs, CDM8 plasmids containing cDNAs encoding CD7 as described by Aruffo and Seed, (EMBO Jour. 6:3313-3316 (1987)), incorporated by reference herein, were also used.

mAbs. Murine monoclonal antibodies (mAbs) 9.3 (anti-CD28) and G19-4 (anti-CD3), G3-7 (anti-CD7), BB-1 (anti-B7 antigen) and rat mAb 187.1 (anti-mouse K chain) have been described previously (Ledbetter et al., Proc. Natl. Acad. Sci. 84:1384-1388 (1987); Ledbetter et al., Blood 75:1531 (1990); Yokochi et al., supra) and were purified from ascites before use. The hybridoma producing mAb OKT8 was obtained from the ATCC, Rockville, MD, and the mAb was also purified from ascites before use. mAb 4G9 (anti-CD19) was provided by Dr. E. Engleman, Stanford University, Palo Alto, CA). Purified human-mouse chimeric mAb L6 (having human Cγ1 Fc portion) was a gift of Dr. P. Fell and M. Gayle (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA).

Immunostaining and FACS^R Analysis. Prior to staining, COS or CHO cells were removed from their culture vessels by incubation in PBS containing 10 mM EDTA. Cells were first incubated with mAbs or Ig fusion proteins at 10 µg/ml in DMEM containing 10% FBS for 1-2 hr at 4° C. Cells were then washed, and incubated for an additional 0.5-2 hrs at 4° C with FITC-conjugated goat anti-mouse immunoglobulin or with FITC-conjugated goat anti-human Ig C γ serum (both from Tago, Burlingame, CA). When binding of both mAbs and Ig fusion proteins were measured in the same experiment, FITC-conjugated anti-mouse and anti-human second step reagents were mixed together before use. Fluorescence on a total of 10,000 cells was then analyzed by FACS^R.

Peripheral Blood Lymphocyte Separation and Stimulation. Peripheral blood lymphocytes (PBLs) were isolated by centrifugation through Lymphocyte Separation Medium (Litton Bionetics, Kensington, MD). Alloreactive T cells were isolated by stimulation of PBL in a primary mixed lymphocyte reaction (MLR). PBL were cultured at 10^6/ml irradiated (5000 rad) T51 LCL. EBV-transformed lymphoblastoid cell lines (LCL), PM (Bristol-Myers Squibb Co.) and T51 (Bristol-Myers Squibb Co.) were maintained in RPMI supplemented with 10% FBS. After 6 days, alloreactive "blasts" cells were cryopreserved. Secondary MLR were conducted by culturing thawed alloreactive blasts together with fresh irradiated T51 LCL in the presence and absence of mAbs and Ig fusion proteins. Cells were cultured in 96 well flat bottom plates (4×10^4 alloreactive blasts and 1×10^4 irradiated T51 LCL cells/well, in a volume of 0.2 ml) in RPMI containing 10% FBS. Cellular proliferation of quadruplicate cultures was measured by uptake of [^{3}H]-thymidine during the last 6 hours of a 2-3 day culture.

PHA-activated T cells were prepared by culturing PBLs with 1 µg/ml PHA (Wellcome, Charlotte, NC) for five days, and one day in medium lacking PHA. Viable cells were collected by sedimentation through Lymphocyte Separation Medium before use. Cells were stimulated

with mAbs or transfected CHO cells for 4-6 hr at 37°C, collected by centrifugation and used to prepare RNA.

$CD4^+$ T cells were isolated from PBLs by separating PBLs from healthy donors into T and non-T cells using sheep erythrocyte rosetting technique and further separating T cells by panning into $CD4^+$ cells as described by Damle et al., J. Immunol. 139:1501 (1987), incorporated by reference herein.

B cells were also purified from peripheral blood by panning as described by Wysocki and Sato, Proc. Natl. Acad. Sci. 75:2844 (1978), incorporated by reference herein, using anti-CD19 mAb 4G9. To measure T_h-induced Ig production, 10^6 $CD4^+$ T cells were mixed with 10^6 $CD19^+$ B cells in 1 ml of RPMI containing 10% FBS. Following culture for 6 days at 37°C, production of human IgM was measured in the culture supernatants using solid phase ELISA as described by Volkman et al., Proc. Natl. Acad. Sci. USA 78:2528 (1981), incorporated by reference herein.

Briefly, 96-well flat bottom microtiter ELISA plates (Corning, Corning, NY) were coated with 200 µl/well of sodium carbonate buffer (pH 9.6) containing 10 µg/ml of affinity-purified goat anti-human IgG or IgM antibody (Tago, Burlingame, CA), incubated overnight at 4°C, and then washed with PBS and wells were further blocked with 2% BSA in PBS (BSA-PBS).

Samples to be assayed were added at appropriate dilution to these wells and incubated with 200 µl/well of 1:1000 dilution of horseradish peroxidase (HRP)-conjugated $F(ab')_2$ fraction of affinity-purified goat anti-human IgG or IgM antibody (Tago). The plates were then washed, and 100 µl/well of o-phenylenediamine (Sigma Chemical Co., St. Louis, MO) solution (0.6 mg/ml in citrate-phosphate buffer with pH 5.5 and 0.045% hydrogen peroxide). Color development was stopped with 2 N sulfuric acid. Absorbance at 490 nm was measured with an automated ELISA plate reader.

Test and control samples were run in triplicate and the values of absorbance were compared to those obtained with known IgG or IgM standards run simultaneously with the supernatant samples to generate the standard curve using which the concentrations of Ig in the culture supernatant were quantitated. Data are expressed as ng/ml of Ig ± SEM of either triplicate or quadruplicate cultures.

Immunoprecipitation Analysis and SDS PAGE. Cells were surface-labeled with ^{125}I and subjected to immunoprecipitation analysis. Briefly, PHA-activated T cells were surface-labeled with ^{125}I using lactoperoxidase and H_2O_2 as described by Vitetta et al., J. Exp. Med. 134:242 (1971), incorporated by reference herein. SDS-PAGE chromatography was performed on linear acrylamide gradients gels with stacking gels of 5% acrylamide. Gels were stained with Coomassie Blue, destained, and photographed or dried and exposed to X ray film (Kodak XAR-5).

Binding Assays. B7Ig was labeled with ^{125}I to a specific activity of approximately 2 x 10^6 cpm/pmole. Ninety-six well plastic dishes were coated for 16-24 hrs with a solution containing CTLA4Ig (0.5 µg in a volume of 0.05 ml of 10 mM Tris, pH 8). Wells were blocked with binding buffer (DMEM containing 50 mM BES (Sigma Chemical Co.), pH 6.8, 0.1% BAS, and 10% FCS) before addition of a solution (0.09 ml) containing ^{125}I B7Ig (approximately 5 x 10^5 cpm) in the presence or absence of competitor. Following incubation for 2-3 hrs at 23° C, wells were washed once with binding buffer, and four times with PBS. Bound radioactivity was then solubilized by addition of 0.5N NaOH, and quantified by gamma counting.

Binding to B7Ig. The functional activity of the OMCTLA4 construct encoding the complete human CTLA4 DNA gene, is shown in the experiment shown in Figure 4. COS cells were transfected with expression plasmids CD7, OMCD28 and OMCTLA4 as described above. Forty-eight hours following transfection, cells were collected and incubated with medium only (no addition) or with mAbs 9.3, B7Ig,

CD5Ig or G3-7. Cells were then washed and binding was detected by a mixture of FITC-conjugated goat anti-mouse Ig and FITC-conjugated goat anti-human Ig second step reagents. Transfected cells were tested for expression of the appropriate cell surface markers by indirect immunostaining and fluorescence was measured using FACS^R analysis as described above.

As shown in Figure 4, mAb 9.3 bound to CD28-transfected COS cells, but not to CTLA4-transfected cells. In contrast, the B7Ig fusion protein (but not control CD5Ig fusion protein) bound to both CD28- and CTLA4-transfected cells. CD7-transfected COS cells bound neither mAb 9.3 nor either of the fusion proteins. This indicates that CD28 and CTLA4 both bind the B cell activation antigen, B7. Furthermore, mAb 9.3 did not detectably bind CTLA4.

Binding of CTLA4Ig on B7 Positive CHO cells. To further characterize the binding of CTLA4Ig and B7, the binding activity of purified CTLA4Ig on B7$^+$ CHO cells and on a lymphoblastoid cell line (PM LCL) was measured in the experiment shown in Figure 5. Amplified transfected CHO cell lines and PM LCLs were incubated with medium only (no addition) or an equivalent concentration of human IgCγ1-containing proteins (10 µg/ml) of CD5Ig, CD28Ig or CTLA4Ig. Binding was detected by FACS^R following addition of FITC-conjugated goat anti-human Ig second step reagents. A total of 10,000 stained cells were analyzed by FACS^R.

As shown in Figure 5, CD28Ig bound to B7$^+$ CHO cells but not to PM LCL, a cell line which expresses relatively low levels of the B7 antigen (Linsley et al., supra, 1990). CTLA4Ig bound more strongly to both cell lines than did CD28Ig, suggesting that it bound with higher affinity. Neither CD28Ig nor CTLA4Ig bound to CD28$^+$ CHO cells.

Affinity of Binding of CTLA4Ig and B7Ig. The apparent affinity of interaction between CTLA4Ig and B7Ig was then measured using a

solid phase competition binding assay. Ninety-six well plastic dishes were coated with CTLA4Ig as described above. B7Ig was radiolabeled with ^{125}I (5 X 10^5 cpm, 2 X 10^6 cpm/pmole), and added to a concentration of 4 nM in the presence of the indicated concentrations (Figure 6) of unlabeled chimeric mAb L6, mAb 9.3, mAb BB-1 or B7Ig. Plate-bound radioactivity was determined and expressed as a percentage of radioactivity bound to wells treated without competitor (28,300 cpm). Each point represents the mean of duplicate determinations; replicates generally varied from the mean by $\leq$ 20%. Concentrations were calculated based on a M_r of 75,000 per binding site for mAbs and 51,000 per binding site for B7Ig.

As shown in Figure 6, only mAb BB-1 and unlabeled B7Ig competed significantly for ^{125}I-B7Ig binding (half maximal effects at approximately 22 nM and approximately 175 nM, respectively). Neither chimeric mAb L6, nor mAb 9.3 competed effectively at the concentrations tested. In other experiments, the concentrations of mAb 9.3 used were sufficient to inhibit binding of ^{125}I-B7Ig to immobilized CD28Ig or to cell surface expressed CD28 by $\geq$ 90%.

When the competition data from Figure 6 were plotted in a Scatchard representation, a dissociation constant, K_d, of approximately 12 nM was calculated for binding of ^{125}I-B7 to immobilized CTLA4Ig (Figure 7). This value is approximately 20 fold lower than the previously determined K_d of binding between ^{125}I-B7Ig and CD28 (approximately 200 nM) (Linsley et al, (1991), supra) indicating that CTLA4 is a higher affinity receptor for the B7 antigen than CD28 receptor.

To identify the molecule(s) on lymphoblastoid cells which bound CTLA4Ig (Figure 7), ^{125}I-surface labeled cells were subjected to immunoprecipitation analysis (Figure 8). $B7^+$ CHO and PM LCL cells were surface-labeled with ^{125}I, and extracted with a non-ionic detergent solution as described above. Aliquots of extracts containing approximately 1.5 X 10^7 cpm in a volume of 0.1 ml were subjected to immunoprecipitation analysis as described above with

no addition, or 2 μg each of CD28Ig, CTLA4Ig or CD5Ig. Washed immunoprecipitates were then analyzed by SDS-PAGE (10-20% acrylamide gradient) under reducing conditions. The gel was then dried and subjected to autoradiography. The left panel of Figure 8 shows an autoradiogram obtained after a 1 day exposure. The right panel of Figure 8 shows an autoradiogram of the same gel after a 10 day exposure. The autoradiogram in the center panel of Figure 8 was also exposed for 10 days. Positions of molecular weight standard are also indicated in this figure.

As shown by Figure 8, a diffusely migrating (M_r approximately 50,000 - 75,000; center at approximately 60,000) radiolabeled protein was immunoprecipitated by CTLA4Ig, but not by CD28Ig or CD5Ig. This molecule co-migrated with B7 immunoprecipitated from $B7^+$ CHO cells by CTLA4Ig, and much more weakly, by CD28Ig. These findings indicate that CTLA4Ig binds a single protein on lymphoblastoid cells which is similar in size to the B7 antigen.

Inhibition of Immune Responses In Vitro by CTLA4Ig

Inhibition of Proliferation. Previous studies have shown that the anti-CD28 mAb, mAb 9.3, and the anti-B7 mAb, mAb BB-1, inhibit proliferation of alloantigen specific T_h cells, as well as immunoglobulin secretion by alloantigen-presenting B Cells (Damle, et al., Proc. Natl. Acad. Sci. 78:5096 (1981); Lesslauer et al., Eur. J. Immunol. 16:1289 (1986)). Because CTLA4 is a high affinity receptor for the B7 antigen as demonstrated herein, soluble CTLA4Ig was tested for its ability to inhibit these responses. The effects of CTLA4Ig on T cell proliferation were examined in the experiment shown in Figure 9.

Primary mixed lymphocyte reaction (MLR) blasts were stimulated with irradiated T51 lymphoblastoid cells (LC) in the absence or presence of concentrations of murine mAb 9.3 Fab fragments, or B7Ig, CD28Ig or CTLA4Ig immunoglobulin Cγ fusion proteins. Cellular

proliferation was measured by [^{3}H]-thymidine incorporation after 4 days and is expressed as the percentage of incorporation by untreated cultures (21,000 cpm). Figure 9 shows the means of quadruplicate determinations (SEM $\leq$ 10%).

As shown in Figure 9, CTLA4Ig inhibited the MLR reaction in a dose-dependant fashion by a maximum of > 90% with a 1/2 maximal response at approximately 30 ng/ml (approximately 0.8 nM). The Fab fragment of mAb 9.3, which previously was shown to be a more potent inhibitor of MLR than whole mAb 9.3 (Damle et al., J. Immunol. 140:1753-1761 (1988)), also inhibited the MLR, but at higher concentrations (approximately 800 ng/ml or approximately 30 nM for 1/2 maximal response). B7Ig and CD28Ig did not significantly inhibit the MLR even at higher concentrations. In another experiment, addition of B7Ig together with CTLA4Ig partially overcame the inhibition of MLR by CTLA4Ig, indicating that the inhibition was specifically due to interactions with B7 antigen.

Inhibition of Immunoglobulin Secretion. The effects of CTLA4Ig on helper T cell (T_h)-induced immunoglobulin secretion were also examined (Figure 10). $CD4^+$ T cells were mixed with allogeneic $CD19^+$ B cells in the presence or absence of the indicated immunoglobulin molecules as described above. Murine mAbs OKT8, 9.3 and BB-1 were added at 20 µg/ml, and Ig fusion proteins at 10 µg/ml. After 6 days of culture, concentrations of human IgM (SEM < 5%) in culture supernatants were determined by enzyme immunoassay (ELISA) as described above. IgM production by B cells cultured in the absence of $CD4^+$ T cells was 11 ng/ml.

As shown in Figure 10, $CD4^+$ T cells stimulated IgM production by allogenic $CD19^+$ B Cells (in the absence of $CD4^+$ T cells, IgM levels were reduced by 93%). mAbs 9.3 and BB-1 significantly inhibited T_h-induced IgM production (63% and 65% inhibition, respectively). CTLA4Ig was even more effective as an inhibitor (89% inhibition) than were these mAbs. Inhibition by control Ig molecules, mAb OKT8

and CD5Ig, was much less (≤ 30% inhibition). None of these molecules significantly inhibited Ig production measured in the presence of Staphylococcal aureus enterotoxin B. Similar results were obtained with $CD4^+$ T cells and B cells derived from other donors. These results indicate that the inhibition by CTLA4Ig is specific.

The above data also demonstrate that the CTLA4 and CD28 receptors are functionally as well as structurally related. Like CD28, CTLA4 is also a receptor for the B cell activation antigen, B7. CTLA4Ig bound ^{125}I-B7 with an affinity constant, K_d, of approximately 12 nM, a value some 20 fold higher than the affinity between CD28 and B7Ig (approximately 200 nM). Thus, CTLA4 and CD28 may be thought of as high and low affinity receptors, respectively, for the same ligand, the B7 antigen.

The apparent affinity between CD28 and B7 is similar to the affinity reported for binding of soluble alloantigen to the T cell receptor of a murine T cell hybridoma (approximately 100 nM; Schnek et al., Cell 56:47 (1989)), and is higher affinity than interactions between CD2 and LFA3 (Recny et al., J. Biol. Chem. 265:8542 (1990)), or CD4 and MHC class II molecules (Clayton et al., Nature 339:548 (1989)). The apparent affinity constant, K_d, between CTLA4 and B7 is even greater, and compares favorably with higher affinity mAbs (K_d 2-10,000 nM; Alzari et al., Ann. Rev. Immuno. 6:555 (1988)). The K_d between CTLA4 and B7 is similar to or greater than K_d values of integrin receptors and their ligands (10-2000 nM; Hautanen et al., J. Biol. Chem. 264:1437-1442 (1989); Di Minno et al., Blood 61:140-148 (1983); Thiagarajan and Kelley, J. Biol. Chem. 263:035-3038 (1988)). The affinity of interaction between CTLA4 and B7 is thus among the highest yet reported for lymphoid adhesion systems.

These results demonstrate the first expression of a functional protein product of CTLA4 transcripts. CTLA4Ig, a fusion construct

containing the extracellular domain of CTLA4 fused to an IgCγ1 domain, forms a disulfide-linked dimer of M_r approximately 50,000 subunits (Figure 1). Because no interchain disulfides would be predicted to form in the Ig portion of this fusion, it seems likely that cysteines from CTLA4 are involved in disulfide bond formation. The analogous CD28Ig fusion protein (Linsley et al, supra, 1991) also contains interchain disulfide linkage(s). These results suggest that CTLA4 receptor, like CD28 (Hansen et al., Immunogenetics 10:247-260 (1980)), exists on the T cell surface as a disulfide linked homodimer. Although CD28 and CTLA4 are highly homologous proteins, they are immunologically distinct, because the anti-CD28 mAb, mAb 9.3, does not recognize CTLA4 (Figures 4 and 5).

It is not known whether CTLA4 can activate T cells by a signalling pathway analogous to CD28. The cytoplasmic domains of murine and human CTLA4 are identical (Dariavach et al., supra 1988), suggesting that this region has important functional properties. The cytoplasmic domains of CD28 and CTLA4 also share homology, although it is unclear if this is sufficient to impart similar signaling properties to the two molecules.

CTLA4Ig is a potent inhibitor of in vitro lymphocyte functions requiring T cell and B cell collaboration (Figures 9 and 10). These findings, together with previous studies, indicate the fundamental importance of interactions between B7 antigen and its counter-receptors, CD28 and/or CTLA4, in regulating both T and B lymphocyte responses. CTLA4Ig should be a useful reagent for future investigations on the role of these interactions during immune responses. CTLA4Ig is a more potent inhibitor of in vitro lymphocyte responses than either mAb BB-1 or mAb 9.3 (Figures 9 and 10). The greater potency of CTLA4Ig over mAb BB-1 is most likely due to the difference in affinities for B7 between these molecules (Figure 6). CTLA4Ig is also more potent than mAb 9.3, probably because, unlike the mAb, it does not also have direct stimulatory effects on T cell proliferation (June et al., Immunology Today

11:211 (1989)) to counteract its inhibitory effects. The immunosuppressive effects of CTLA4Ig *in vitro* suggest that future investigations are warranted into possible therapeutic effects of this molecule for treatment of autoimmune disorders involving aberrant T cell activation or Ig production.

As will be apparent to those skilled in the art to which the invention pertains, the present invention may be embodied in forms other than those specifically disclosed above without departing from the spirit or essential characteristics of the invention. The particular embodiments of the invention described above, are, therefore, to be considered as illustrative and not restrictive. The scope of the present invention is as set forth in the appended claims rather than being limited to the examples contained in the foregoing description.

EXAMPLE 5

Female BALB/c (H-2^d) and C57BL/6 (H-2^d)mice, 6 to 8 wk. of age were obtained from The Jackson Laboratory (Bar Harbor, ME).

Human pancreatic islets cells were purified after collagenase digestion as described (C. Ricordi et al. Transplantation 52:519 (1991); A. G. Tzakis et al. Lancet 336:402 (1990); C. Ricordi, P. E. Lacy, E. H. Finke, B. J. Olack, D. W. Scharp, Diabetes 37:413 (1988)).

B6 or B10 mice, treated with streptozocin (175 mg per kilogram of body weight) 3 to 5 days before transplant and exhibiting nonfasting plasma glucose levels of greater than 280 mg/dl (with the majority over 300 mg/ml), were used as recipients.

Each animal received approximately 800 fresh human islets of 150 μm in diameter beneath the left renal capsule (D. Faustman and C. Coe, Science 252:1700 (1991); Y. J. Zeng et al. Transplantation 53:277

(1992)). Treatment was started immediately after transplantation.

Control animals were treated with PBS (solid lines) or L6 (dotted lines) at 50 μg every other day for 14 days immediately after transplantation (Figure 11A). Islet transplants were considered rejected when glucose levels were greater than 250 mg/dl for three consecutive days. Animals treated with PBS (n = 14) and L6 (n = 8) had mean graft survivals of 5.6 and 6.4 days, respectively.

Animals were treated with 10 μg of CTLA4Ig for 14 consecutive days immediately after transplant (n = 7) (Figure 11B). Three out of seven animals maintained their grafts for >80 days. The remaining four animals had a mean graft survival of 12.75 days.

Animals were treated with 50 μg of CTLA4Ig every other day for 14 days immediately after human islet transplantation (Figure 11C). All animals (n = 12) treated with this dose maintained grafts throughout the analysis (Figure 11C). Selected mice were nephrectomized on days 21 and 29 after the transplant to assess the graft's function (Figure 11C).

Histology was performed on kidneys transplanted with human islet cells (Figures 12A, 12B, 12C, 12D). The slides were analyzed blindly.

Hematoxylin and eosin staining of a control human islet grafted mouse 29 days after transplantation showed a massive lymphocyte infiltration (Figure 12A). The same tissue, stained for insulin, showed no detectable insulin production (Figure 12B).

Histological examination of tissue from a CTLA4Ig-treated mouse 21 days after transplant showed intact islets under the kidney capsule with very few lymphocytes infiltrating the transplanted tissue (Figure 12C). The tissue was stained with hematoxylin and eosin. The same tissue from the CTLA4Ig-treated mouse, stained for

insulin, showed the production of insulin by the grafted islets (Figure 12D). Similar results were observed in graft tissue examined at later time points. The upper, middle, and lower arrowheads identify the kidney capsule, islet transplant, and kidney parenchyma, respectively.

In the histopathology assay all tissues were fixed in 10% buffered formalin and processed, and 5-μm sections were stained either with hematoxylin and eosin or for insulin with the avidin-biotin-peroxidase method (S. M. Hsu, L. Raine, H. Fanger, J. Histochem, Cytochem, 29:577 (1981)). Magnification was x 122.

In Figure 13 streptozotocin-treated animals were transplanted as described hereinabove for Figure 11. The mice were treated either with PBS (dotted lines) or with MAb to human B7 (solid lines) at a dose of 50 μg every other day for 14 days (Figure 13). Control animals (treated with PBS) (n = 3) had a mean graft survival of 3.5 days, whereas anti-B7-treated animals (n = 5) maintained grafts from 9 to >50 days (Figure 13).

In Figure 14 normal glycemic, CTLA4Ig-treated, transplanted mice (dotted lines) were nephrectomized on day 44 after transplant and immediately retransplanted with either 1000 first party donor islets (dotted lines, solid circles) or 1000 second party islets (dotted lines, open circles) beneath the remaining kidney capsule.

These islets, frozen at the time of the first transplant, were thawed and cultured for 3 days before transplant to ensure islet function. B10 mice that had been treated with streptozotocin and exhibited nonfasting glucose levels of greater than 280 mg/dl were used as controls (solid lines) (Figure 14). No treatment was given after transplantation.

Control animals rejected both the first party (solid lines, closed circles) and the second party (solid lines, open circles) islet

grafts by day 4 after transplant (Figure 14). The CTLA4Ig-treated mice retransplanted with second party islets had a mean graft survival of 4.5 days, whereas animals retransplanted with first party donor islets maintained grafts for as long as analyzed (>80 days) (Figure 14).

CTLA4Ig significantly prolongs human islet graft survival in mice in a donor-specific manner thereby providing an approach to immunosuppression

C57BL/6 (B6) or C57BL/10 (B10) mice were treated with streptozotocin to eliminate mouse pancreatic islet B dell function. Diabetic animals were grafted under the kidney capsule, and treatment was started immediately after surgery. Survival of the islet grafts was monitored by the analysis of blood glucose concentrations.

Transplanted control animals, treated with either phosphate-buffered saline (PBS)(n = 14) or L6 (a human IgG1 chimeric MAb; n = 8), had a mean graft survival of 5.6 and 6.4 days, respectively (Figure 11A).

In contrast, islet rejection was delayed in animals treated with CTLA4Ig (10 μg per day for 14 days), with four out of the seven animals exhibiting moderately prolonged mean graft survival (12.75 days), whereas the remaining three animals maintained normal glucose levels for >80 days (Figure 11B). This eventual increase in glucose concentration may be a result of islet exhaustion because no evidence of active cellular rejection was observed.

In the three mice that maintained long-term islet grafts, the transient increase in glucose concentrations around day 21 after the transplant may have represented a self-limited rejection episode consistent with the pharmacokinetics of CTLA4Ig clearance after therapy (P. S. Linsley et al., Science 257:792 (1992)).

In subsequent experiments, the dose of CTLA4Ig was increased to 50 µg per animal every other day for about 14 days. This treatment resulted in 100% of the animals maintaining normal islet function throughout the experiment with no signs of a rejection crisis (Figure 11C).

In order to confirm that insulin production originated from the transplanted islets and not from the native mouse pancreas, we nephrectomized selected animals at days 21 and 29 to remove the islet grafts (Figure 11C). In these animals, glucose concentrations increased to above 350 mg/dl within 24 hours, which indicated that the islet xenograft was responsible for maintaining normal glucose levels. It appears that the blocking of the CD28-B7 interaction inhibits xenogenic islet graft rejection.

The effects of treatment with the soluble receptor, namely CTLAIg fusion protein, were not a result of Fc binding (L6 did not effect graft rejection) or general effects on T cell or B cell function in vivo.

Historical analyses of islet xenograft from control (PBS treated) and CTLA4Ig treated mice were done (Figures 12A, 12B, 12C, 12D). The islet tissue from the control animal demonstrated evidence of immune rejection, with a marked lymphocytic infiltrate into the graft and few remaining islets (Figure 12A).

Immunohistochemical staining showed that insulin-positive cells were present only rarely, and no somatostatin-positive cells were present at all (Figure 12B). In contrast, transplant tissue from the CTLA4Ig-treated mice was devoid of any lymphocytic infiltrate (Figure 12C).

The grafts were intact, with many islets visible. In addition, the B cells observed in the human islet tissue produced human insulin (Figure 12D) and somatostatin.

The human CTLA4Ig used in this study reacts with both murine and human B7. One advantage of the xenogeneic transplant model is the availability of a MAb to human B7 that does not react with mouse B7 (T. Yokochi, R. D. Holly, E. A. Clark, J. Immunol. 128:823 (1982)). Thus, the role of human B7-bearing antigen-presenting cells (APCs) could be directly examined.

The mice were transplanted as described and then treated with 50 µg of MAb to human B7 every other day for 14 days after transplant. This treatment prolonged graft survival in treated mice (9 to >50 days) in comparison to that for control mice (Figure 13). The anti-B7 MAb is unable to block rejection as effectively as CTLA4Ig.

The CTLA4Ig therapy resulted in graft acceptance in the majority of mice. However, the animals may not be tolerant. Transient immunosuppression can lead to permanent islet graft acceptance because of graft adaptation (the loss of immunogenicity as a result of the loss of APC function) (L. Hao, Y. Wang, R. G. Gill, K. J. Lafferty, J. Immunol. 139:4022 (1987); K. J. Lafferty, S. J. Prowse, M. Simeonovic, Annu. Rev. Immunol. 1:143 (1983)).

In order to differentiate between these possibilities, we nephrectomized selected xenografted, CTLA4Ig-treated mice (day 40) and retransplanted them under the remaining kidney capsule with either the original donor islets (first party) or unrelated second party human islets (Figure 14).

Streptozotocin-treated control animals, having never received an islet graft, were also transplanted with either first or second party islets. No treatment after the transplant was given. Control animals rejected the first and second party islets by day 4. The CTLA4Ig-treated animals that had received the second party islets rejected these islets by day 5, whereas animals receiving first party donor islets maintained the grafts for >80 days (Figure 14).

These results suggest that the CTLA4Ig treatment resulted in prolonged donor-specific unresponsiveness to the xenogeneic islets. The ability of the murine immune response to distinguish differences among the human islet donors also supports the direct recognition of the polymorphic MHC products expressed on the human islet cells.

EXAMPLE 6

Female BALB/c ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^d$)mice, 6 to 8 wk. of age were obtained from The Jackson Laboratory (Bar Harbor, ME).

Monoclonal antibody 11B11 is a rat IgG1 anti-murine IL-4 (Ohara, J., and W. E. Paul, 1985, Production of a monoclonal antibody to and molecular characterization of B-cell stimulatory factor-1. Nature 315:333) (Verax (Lebanon, NH)).

BALB/c mice (five per group) were immunized intravenously with 10^8 SRBC alone or together with 200 μg chimeric L6 mAb or human CTLA4Ig fusion protein. The indicated groups were treated 2 hrs. prior to injection of SRBCs by intraperitoneal injection of 2 mls of either rat immunoglobulin or rat anti-murine IL-4 mAb 11B11 at 5 mg/ml. Treatment with chimeric L6 mAb or CTLA4Ig was repeated daily for 4 additional days.

All animals were given intravenous injections of SRBCs (Figure 15) or KLH (Figure 16) on day 46. Specifically, in Figure 15, the closed circle represents mice who were administered with only SRBC at day 0 and day 46. The open circle represents mice administered with only SRBC at day 46. The remaining mice represented in Figure 15 were further administered with SRBC at day 46. In contrast, in Figure 16, the mice were administered with a different immunogen, KLH, at day 46 only.

Serum concentrations of mice measured as having antibodies directed

against SRBCs or KLH were determined by ELISA as described (Linsley et al., Science 1992).

Serum antibody titers were calculated as the dilution giving an A_{450} of five times background. Serum antibody titer values from Figure 15 were determined from pooled sera from five mice per group, while serum antibody titer values from Figure 16 represents mean titers of five individual sera. Arrows indicate an SRBC or KLH injection at day 46.

Figures 15 and 16 show that the immunological response in mice injected concurrently with both CTLA4Ig and anti-IL4 (open triangle) is suppressed in an antigen-specific manner.

Figure 15 shows that there is no rise in serum antibody titer (i.e. no primary or secondary immunological response) in mice injected concurrently with CTLA4Ig and anti-IL4 and injected with SRBC at day 0 and day 46. The combination of CTLA4Ig and anti-IL4 suppresses a primary and secondary immune response and induces long lasting immunological non-responsiveness to SRBC.

Additionally, Figure 15 shows that there is no primary immunological response in mice injected concurrently with CTLA4Ig and the control rat Ig (Cappel, Organontecknika, Palo Alto, CA). However, these mice exhibit a secondary immunological response after injection with SRBC at day 46 (closed triangle, Figure 15).

Figure 16 shows that administration of CTLA4Ig and anti-IL4, followed by a different immunogen, KLH, at day 46 in mice does not suppress a primary immune response to KLH in mice. Instead, these mice exhibited a primary immune response to KLH (open triangle, Figure 16). Thus, mice treated with CTLA4Ig and anti-IL4 exhibited a highly specific immune response depending on the antigen administered therein.

EXAMPLE 7

By site-specific and homolog mutagenesis, we have identified regions in CTLA4Ig which are required for its high avidity binding to B7-1. The following is a description of how to make soluble CTLA4/CD28 hybrid fusion proteins which bind B7.

MATERIALS AND METHODS

Monoclonal antibodies (mAbs). Murine mAb's specific for CTLA4 were prepared and characterized as previously described (Linsley et al. J. Ex. Med., (1992) 176:1595-1604). Antibody 9.3 (anti-CD28) has been described previously ((Hansen et al., Immunogenetics 10:247-260 (1980)).

Cell Culture. The preparation of stably transfected B7-1 positive CHO cells has been previously described (Linsley et al., in J. Exp. Med. 173:721-730 (1991); P. S. Linsley et al., J. Exp. Med. 174:561 (1991)).

Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 0.2mM proline, and 1μM methotrexate. COS cells were grown in DMEM supplemented with 10% FBS. CTLA4Ig was prepared in CHO cells as previously described (Example 2).

CTLA4Ig and CD28Ig site-directed mutant expression plasmids. Site-directed mutagenesis was performed on a vector encoding soluble chimeric form of CTLA4 (CTLA4Ig) in which the extracellular domain of CTLA4 was genetically fused to the hinge and constant regions of a human IgG heavy chain (Example 2). CTLA4Ig site-directed mutants were prepared by encoding the desired mutation in overlapping oligonucleotide primers and generating the mutants by PCR (Ho et al., 1989, supra.) using the CTLA4Ig plasmid construct as a template.

INS D19 Six mutants were prepared which encoded substitutions to alanine in the highly conserved hexapeptide 98MYPPPY103 forming part of the putative CDR3-like domain (Figures 17 and 22) (Ho et al., 1989, supra.). These mutants are described in Table II.

INS D20 In addition, two mutants encoding the residues P103A and Y104A (MYPPAY and MYPPPA, respectively) from the CD28Ig 99MYPPPY104 hexapeptide using CD28Ig as a template were also prepared by the same method. These mutants are also described in Table II.

Primers required for PCR reactions but not for introducing mutations included (1) a CDM8 forward (CDM8FP) primer encoding a complementary sequence upstream of the HindIII restriction site at the 5' end of the CDM8 stuffer region, and (2) a reverse primer (CDM8RP) encoding a complementary sequence downstream of the XbaI site at the 3' end of the CDM8 stuffer region.

INS D21 These primers encoded the following sequences:

CDM8FP:5'-AATACGACTCACTATAGG

CDM8RP:5'-CACCACACTGTATTAACC

PCR conditions consisted of 6 min at 94°C followed by 25 cycles of 1 min at 94°C, 2 min at 55°C and 3 min at 72°C. Taq polymerase and reaction conditions were used as suggested by the vendor (Perkin Elmer Cetus, Emeryville, CA). PCR products were digested with HindIII and XbaI and ligated to HindIII/XbaI-cut CDM8 expression vector.

To confirm that the desired mutations had been inserted and to verify the absence of secondary mutations, each CTLA4Ig mutant fusion protein (an example of a soluble CTLA4 mutant fusion protein) was sequenced by the dideoxy chain termination/extension reaction with Sequenase reagents used according to the manufacturers recommendations (United States Biochemical Corp., Cleveland, OH).

Plasmids were transfected into COS cells (Aruffo et al., Cell 61:1303 (1990)) and the conditioned media was used as a source for the resulting Ig mutant fusion proteins.

CTLA4/CD28Ig hybrid expression plasmids. CTLA4/CD28Ig hybrid scan plasmids encoding the constructs HS2, HS4, HS4-A, HS4-B, and HS5 (Figure 19 and Table I) were prepared by PCR using overlapping oligonucleotide primers designed to introduce CTLA4 sequences into CD28Ig while, at the same time, deleting the equivalent region from CD28. The same CDM8 forward and reverse PCR primers described above were also used.

The following is a list of the CTLA4/CD28 hybrid fusion proteins which were made.

DESIGNTION	FRAMEWORK	MODIFICATIONS
HS1	CTLA4	1-24 OF CD28 97-125 OF CD28
HS2	CD28	1-22 OF CTLA4 96-125 OF CTLA4
HS3	CTLA4	96-125 OF CD28
HS4	CD28	96-123 OF CTLA4
HS4A	CD28	96-113 OF CTLA4
HS4B	CD28	114-123 OF CTLA4
HS5	CD28	25-32 OF CTLA4
HS6	CTLA4	25-32 OF CD28
HS7	CD28	96-123 OF CTLA4 25-32 OF CTLA4
HS8	CD28	25-32 OF CTLA4 96-113 OF CTLA4
HS9	CD28	25-32 OF CTLA4 114-123 OF CTLA4
HS10	CD28	96-123 OF CTLA4 51-58 OF CTLA4
HS11	CD28	25-32 OF CTLA4

		51-58 OF CTLA4
		96-123 OF CTLA4
HS12	CD28	51-58 OF CTLA4
		96-113 OF CTLA4
HS13	CD28	25-32 OF CTLA4
		51-58 OF CTLA4
		96-113 OF CTLA4
HS14	CD28	51-58 OF CTLA4

Each cDNA construct was genetically linked to cDNA encoding the hinge and constant regions of a human IgG1 in order to make soluble chimeras.

A HS6 hybrid was prepared in a similar manner to that described above except that the CDR1-like region in CTLA4Ig was replaced with the equivalent region from CD28Ig.

HS7, HS8, and HS9 constructs were prepared by replacing a ≈350 base-pair HindIII/HpaI 5' fragment of HS4, HS4-A, and HS4-B, respectively, with the equivalent cDNA fragment similarly digested from HS5 thus introducing the CDR1-like loop of CTLA4 into those hybrids already containing the CTLA4 CDR3-like region.

HS10-HS13 constructs are domain homolog mutants which were prepared by introducing the CDR2-like loop of CTLA4Ig into previously constructed homolog mutants. This was done by overlapping PCR mutagenesis whereby primers were designed to introduce CTLA4 CDR2-like sequences into homolog templates while at the same time deleting the equivalent CD28 CDR2-like region from the molecule.

Accordingly, HS4 served as a template to make HS10; HS7 served as a template to make HS11; HS4-A served as a template to make HS12; and HS8 served as a template to make HS13 (Figure 19 and Table I). The CDM8 primers described above were also used in these constructions.

The HS14 hybrid construct was prepared by replacing the CDR2-like loop of CD28 with the equivalent loop from CTLA4Ig (Figure 19 and Table I).

Oligonucleotide primers designed to introduce these changes were used in overlapping PCR mutagenesis identical to that described for other mutants.

PCR reactions and subcloning into CDM8 were performed as described above. Again all mutants were sequenced by the dideoxy chain termination/extension reaction.

Plasmids encoding each of the mutants were transfected into COS cells and the resulting soluble Ig fusion proteins were quantitated in culture media and visualized by Western blot as described in following sections.

Quantitation of the resulting Ig fusion proteins in culture media. Soluble mutant fusion proteins were quantitated in an enzyme immunoassay by determining the amount of Ig present in serum-free COS cell culture media.

Microtiter plates (Immulon2; Dynatech Labs., Chantilly, VA) were coated with 0.5µg/ml goat anti-human IgG (Jackson Immunoresearch Labs., West Chester, PA) for 16-24h at 4°C. Wells were blocked for 1h with specimen diluent (Genetic Systems, Seattle, WA), then washed with PBS containing 0.05% Tween 20 (PBS-Tw).

COS cell culture media containing fusion proteins was added at various dilutions and incubated for 1h at 22°C. Known concentrations of CTLA4Ig were also added to separate wells on each plate for a standard curve.

After washing, horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Tago, Burlingame, CA) diluted 1:12,000 was added and

incubated for 1h at 22°C. Wells were then washed and incubated with 3,3',5,5' tetramethylbenzidine (TMB) substrate (Genetic Systems) for 15 min before stopping the reaction by the addition of 1N H_2SO_4. Optical density was measured at dual wavelengths of 450 and 630nm on a microtiter plate reader (Genetic Systems).

Concentration of mutant Ig fusion protein was determined by comparison with a standard curve of known concentrations of CTLA4Ig.

Immunoprecipitation and Western blot analysis. CTLA4/CD28Ig hybrid fusion proteins present in culture media were adsorbed to protein A-Sepharose by overnight incubation at 4°C. The beads were washed with PBS containing 0.1% Nonidet-P40 (NP40) then SDS PAGE sample buffer was added and the eluted protein was loaded onto an SDS polyacrylamide gel.

Western blot transfer of protein onto nitrocellulose was done by standard procedures. Nitrocellulose membranes were then blocked with PBS containing 0.1% NP40 and 1% non-fat dry milk powder.

After washing in PBS-Tw membranes were incubated with alkaline phosphatase-conjugated goat anti-human IgG (Boehringer Mannheim, Indianapolis, IN) diluted 1:1,000 and incubated for 1h at 22°C. Blots were then washed and developed using standard procedures.

B7 positive CHO cell enzyme immunoassay. The ability of CTLA4Ig mutant fusion proteins, and CTLA4/CD28Ig hybrid fusion proteins to bind B7-1 stably expressed on CHO cells was determined by an enzyme immunoassay.

Round bottom tissue culture treated 96 well microtiter plates (Corning, Corning, NY) were seeded with B7-1 positive CHO cells at 10^3 cells/well. Two days later the confluent cells were fixed in 95% ethanol for 15 min.

After washing with PBS-Tw, mutant Ig fusion proteins were added at various concentrations and incubated for 1h at 4°C. After washing, HRP-conjugated goat anti-human IgG (Tago) diluted 1:10,000 was added and incubated for 1h at 22°C.

Wells were then washed and TMB substrate added as above and allowed to react for 30 min before stopping the reaction with 1N H_2SO_4. Absorbance of the wells was measured at 450nm.

CD28Ig site-directed mutant fusion protein binding assay. Site-directed mutant fusion proteins of CD28Ig were assayed for their ability to bind to B7-1 by an indirect enzyme immunoassay.

Wells of ELISA plates were coated with a chimeric fusion protein containing the extracellular domain of human B7-1 fused to a mouse IgG1 Fc region, at 5µg/ml for 16h at 4°C. Wells were blocked for 1h with specimen diluent (Genetic Systems) then washed with PBS-Tw. COS cell culture media containing known concentrations of mutant fusion protein was added at various concentrations and incubated for 1h at 22°C.

Known concentrations of CD28Ig were also added to separate wells on each plate. After washing, HRP-conjugated goat anti-human IgG (Tago) diluted 1:10,000 was added and incubated for 1h at 22°C. TMB substrate was added and optical densities read as described for quantitation of Ig fusion proteins in culture media.

mAb binding to Ig fusion proteins. The ability of anti-CTLA4 mAb's and the anti-CD28 mAb 9.3 to bind CTLA4/CD28Ig hybrid fusion proteins and CTLA4Ig mutant fusion proteins was assessed by an enzyme immunoassay.

Wells of microtiter plates (Immulon 2) were coated with 0.5µg/ml of goat anti-human IgG (Jackson) for 16-24h at 4°C. Plates were blocked for 1h with specimen diluent (Genetic Systems), washed with

PBS-Tw, then incubated with the Ig fusion proteins for 1h at 22°C. After washing, wells were incubated with mAb at 1µg/ml for 1h at 22°C.

After further washing, HRP-conjugated goat anti-mouse Ig (Tago) diluted 1:10,000 was added and incubated for 1h at 22°C. TMB substrate was added and optical density measured as described above.

CTLA4 molecular model. An approximate three-dimensional model of the CTLA4 extracellular domain was generated based on the conservation of consensus residues of IGSF variable-like domains.

Using such IGSF consensus residues as "anchor points" for sequence alignments, CTLA4 residues were assigned to the A, B, C, C', C", D, E, F, G strands of an Ig variable fold (Williams/Barclay, 1988, supra.) and the connecting loop regions (Figure 22).

The CTLA4 model was built (InsightII, Discover, Molecular Modeling and Mechanics Programs, respectively, Biosym Technologies, Inc., San Diego) using the variable heavy chain of HyHEL-5 (Sheriff et al., 1987 PNAS 84:8075-8079) as template structure. Side-chain replacements and loop conformations were approximated using conformational searching (Bruccoleri et al., 1988 335:564-568).

Several versions of the model with modified assignments of some residues to β-strands or loops were tested using 3D-profile analysis (Lüthy et al., 1992, Nature 336:83-85) in order to improve the initial alignment of the CTLA4 extracellular region sequence with an IGSF variable fold.

RESULTS

Construction and binding activity of CTLA4Ig and CD28Ig mutant fusion proteins. A sequence alignment of various homologues of

CD28 and CTLA4 is demonstrated in Figure 17. In Figure 17, sequences of human (H), mouse (M), rat (R), and chicken (Ch) CD28 are aligned with human and mouse CTLA4. Residues are numbered from the mature protein N-terminus with the signal peptides and transmembrane domains underlined and the CDR-analogous regions noted. Dark shaded areas highlight complete conservation of residues while light shaded areas highlight conservative amino acid substitutions in all family members.

Regions of sequence conservation are scattered throughout the extracellular domains of these proteins with the most rigorous conservation seen in the hexapeptide MYPPPY motif located in the CDR3-like loop of both CTLA4 and CD28 (Figure 17). This suggests a probable role for this region in the interaction with a B7 antigen, e.g., B7-1 and B7-2.

To test this possibility, site-directed alanine scanning mutations were introduced into this region of CTLA4Ig using PCR oligonucleotide primer-directed mutagenesis thereby resulting in CTLA4Ig mutant fusion proteins. Similarly two alanine mutations were introduced into the CD28Ig MYPPPY motif thereby resulting in CD28Ig mutant fusion proteins.

All cDNA constructs were sequenced to confirm the desired mutations before transfection into COS cells. The concentrations of mutant Ig fusion proteins in serum-free COS cell culture media were determined by an Ig quantitation assay.

The ability of each CTLA4Ig mutant fusion protein to bind to B7-1 expressed on stably transfected CHO cells was then determined by an indirect cell binding immunoassay. Binding of CD28Ig mutant fusion proteins to B7-1 was assessed by an indirect enzyme immunoassay. Each of these assays are described in Materials and Methods.

Mutagenesis of each residue of the CTLA4Ig MYPPPY motif to Ala had

a profound effect on binding to B7-1 as shown in Figure 18. Figure 18 shows that mutations in the MYPPPY motif of CTLA4Ig and CD28Ig disrupt binding to B7-1. Site-directed mutant Ig fusion proteins were produced in transiently transfected COS cells, quantitated and tested for their ability to bind to B7-1.

In Figure 18 fusion protein quantitations were repeated at least twice with replicate determinations. Specifically, Figure 18 shows that CTLA4Ig mutants bind to stably transfected, ethanol-fixed B7-1+ CHO cells grown to confluency in ELISA tissue culture plates. Binding data is expressed as the average of duplicate wells and is representative of at least two experiments.

Y99A and P101A mutants bound to B7-1 but with considerably reduced ability relative to wild-type CTLA4Ig. In contrast, the mutants M98A, P100A, P102A and Y103A showed an almost complete loss of binding. Furthermore, the CD28Ig MYPPPY mutants P103A and Y104A did not display detectable binding to B7-1 immobilized on wells of ELISA plates (Figure 18b).

B7-1 transfected CHO cells which were incubated with CTLA4Ig mutant fusion protein, labeled with anti-human FITC, and assayed using a FACSCAN showed equivalent results. These results clearly demonstrate a critical role for the MYPPPY motif in both CTLA4Ig and CD28Ig binding to B7-1.

Characterization of CTLA4/CD28Ig hybrid fusion proteins. Since the MYPPPY motif is common to both CTLA4Ig and CD28Ig, it alone cannot account for the observed differences in binding to B7-1 seen with CTLA4Ig and CD28Ig. The contribution of less well conserved residues to high avidity binding B7-1 was assessed using a series of homolog mutants.

The three CDR-like regions of CD28 were replaced in various combinations with the equivalent regions from the CTLA4

extracellular domain (Figure 19 and Table I). Figure 19 is a map of CTLA4/CD28Ig mutant fusion proteins showing % binding activity to B7-1+ CHO cells relative to CTLA4-Ig. Conserved cysteine residues (C) are shown at positions 22, 93 and 121 respectively (CTLA4 numbering). Also shown is the position of the MYPPPY motif. Open areas represent CD28 sequence; filled areas represent CTLA4 sequence; cross-hatched areas represent beginning of IgG Fc (also refer to Table I). Percent binding activities were determined by comparing binding curves (Figure 20a/b) relative to CTLA4-Ig and finding the concentration of a mutant required to give the same O.D. as that found for CTLA4-Ig. The ratio of mutant protein to CTLA4-Ig concentration at a particular O.D. was then expressed as % binding activity. At least two A450 readings were taken from the linear part of the CTLA4-Ig binding curve and the average % binding activity determined.

A total of 14 hybrid cDNA constructs were prepared, sequenced, and transfected into COS cells. Concentrations of Ig fusion proteins in serum-free culture media were determined and their electrophoretic mobility compared by SDS-PAGE including Western blotting analysis.

Under reducing conditions each chimeric protein migrated with a relative molecular mass ranging between that of CTLA4Ig (Mr-50kDa) and CD28Ig (Mr-70kDa) depending on the size of the exchanged region.

Under non-reducing conditions the proteins migrated primarily between 100-140kDa indicating that these fusion proteins existed as disulfide-linked dimers despite mutagenesis of the cysteine residues in the hinge region of the Fc.

Since four of the five conserved cysteine residues in CTLA4 and CD28 are thought to be involved in intrachain disulfide bonds, dimerization of the fusion proteins was therefore most likely

attributable to the fifth conserved cysteine residue at position 121 in CTLA4 (position 123 in CD28).

Binding of CTLA4/CD28Ig hybrid fusion proteins to B7-1. The hybrid fusion proteins were tested for their ability to bind to B7-1 by the same indirect cell binding immunoassay used to assay the site-specific CTLA4Ig and CD28Ig mutant fusion proteins.

Under these conditions the binding between CD28Ig and B7-1 is barely detectable (Figures 20a/b). However, replacing residues 97 to 125 (the CDR3-like extended region) of CD28 with the corresponding residues of CTLA4 resulted in an approximately two and a half orders of magnitude increase in binding of the CD28Ig analog to B7-1 (Figure 20a/b). Figure 20a/b shows that CTLA4/CD28Ig mutant fusion proteins demonstrate involvement of CDR-analogous regions in high avidity binding to B7-1 CHO cells. Mutants were assayed as described in figure 2. Data is expressed as the average of duplicate wells and is representative of at least three experiments. From these curves % binding activity relative to CTLA4-Ig was determined as explained and shown in Figure 19.

Binding to B7-1 by this construct, termed HS4 (Figure 19), is approximately five fold less than wild type CTLA4Ig. The HS2 hybrid which includes additional N-terminal residues of CTLA4 (amino acids 1-22), did not improve the ability of the hybrid molecule to bind to B7-1 relative to HS4.

The HS6 construct which represents the CTLA4Ig sequence except that it contains the CDR1-like region of CD28 (residues 25-32), bound similarly. However, the additional inclusion of the CTLA4 CDR1-like region (residues 25-32) into the HS4 construct (termed HS7), showed further improved binding so that the binding affinity is approximately 44% of CTLA4Ig (Figure 19).

In contrast, inclusion of the CDR2-like region of CTLA4 (residues

51-58) into HS4 (construct HS10), did not further increase binding (Figure 19). A similar result was found for construct HS11 which had all three CDR-like region sequences of CTLA4 included into CD28Ig. The HS5 hybrid which contained only the CDR1-like domain of CTLA4 bound at very low levels.

The CTLA4/CD28Ig hybrid HS4-A encoded CTLA4Ig residues 96-113 in the C-terminally extended CDR3-like region; nine CTLA4 derived residues fewer than HS4 (Figure 19 and Table I). HS4-A bound B7-1 CHO cells less well than HS4 (Figures 19 and 20b). However, addition of the CTLA4 CDR1-like loop (HS8 hybrid), increased B7-1 binding from about 2% to nearly 60% of wild type binding.

On the other hand, addition of the CTLA4 CDR2-like loop into HS4-A (HS12) did not increase binding relative to HS4-A; neither did addition of all three CTLA4 CDR-like regions (HS13, Figure 19).

Another hybrid called HS4-B, encoded the CD28 CDR3-like region including the MYPPPY motif followed by CTLA4 residues 114-122 (Table I and Figure 19).

HS4-B and HS4-A displayed similar binding to B7-1. Unlike HS4-A, however, the inclusion of the CTLA4 CDR1-like loop into HS4-B (HS9) did not improve binding (Figure 19), suggesting that residues immediately adjacent to the CTLA4Ig MYPPPY motif were important determinants in high avidity binding.

Monoclonal antibody binding to CTLA4/CD28Ig hybrid fusion proteins. The structural integrity of each hybrid fusion protein was examined by assessing their ability to bind mAb's specific for CTLA4 or CD28 in an enzyme immunoassay. The CTLA4 specific mAb's 7F8, 11D4 and 10A8 block ligand binding (Linsley et al. (1992) supra.).

These antibodies bound to each of the CTLA4Ig mutant fusion proteins except 11D4 which failed to bind to P100A and P102A

(Table II). Since 7F8 and 10A8 bound to these mutants, the lack of binding by 11D4 can probably be attributed to mutagenesis perturbing the epitope recognized by 11D4.

Conversely, each antibody failed to bind to any of the homolog scan hybrid fusion proteins except 7F8 which bound to HS6, and 11D4 which bound weakly to HS8. As many of these homolog hybrid fusion proteins were, to some extent, able to bind to B7-1, it is likely that lack of binding by the antibodies was due to disruption of conformational epitopes formed by spatially adjacent but non-linear sequences.

The CD28 specific mAb 9.3 (Linsley et al. (1992) supra.) failed to bind to either of the CD28 site-directed mutant fusion proteins but bound to the hybrid fusion proteins HS4, HS4-A, HS7 and HS8. With HS2, weaker binding was observed. No binding was seen with the HS5 and HS6 constructs.

CTLA4 model. Figure 21 shows a schematic representation of the CTLA4 model. The assignment of CTLA4 residues to CDR-like regions is shown in Figure 17. The CTLA4 model suggests the presence of an additional (non-Ig) disulfide bond between residues Cys49 and Cys67 which supports the similarity of CTLA4 and the Ig variable fold.

The two possible N-linked glycosylation sites in CTLA4 map to solvent exposed positions of the Ig ß-strand framework regions. 3D-profile analysis indicated that the CTLA4 sequence is overall compatible with an Ig V-fold, albeit more distantly related.

Residue Val115 represents the last residue of the CTLA4Ig-like domain. The conformation of the region between Val115 and the membrane-proximal Cys121 which is thought to form the CTLA4 homodimer is highly variable in the CD28 family. The picture that emerges is that CD28 family members mainly utilize residues in two of three CDR-like regions for binding to B7-1.

The MYPPPY motif represents a conserved scaffold for binding which appears to be augmented by its C-terminal extension and which is specifically modulated by the highly variable CDR1-like region. CDR3 and CDR1-like regions are spatially contiguous in Ig-variable folds. The CDR2 like region is spatially distant and does not, in the case of the CD28 family, significantly contribute to the binding to B7-1.

ADP 1 B

TABLE I. CTLA4-Ig/CD28-Ig homolog mutant junction sequences.

MUTANT							
HS1	-22CKYasp27-				-93ckvEVM99-		-123CPSDQE-
HS2	-20fvcKYS25-				-94CKIelm98-		-121cpdDQE-
HS3					-93ckvEVM99-		-123CPSDQE-
HS4					-94CKIelm98-		-121cpdDQE-
HS5	-22CKYasp27-	-30ateFRA35-					-123CPSDQE-
HS6	-22ceySYN27-	-30SREvrv35-					-121cpdDQE-
HS4-A					-94CKIelm98-	-111tqiHVK118-	-123CPSDQE-
HS4-B						-113TIIyvi116-	-121cpdDQE-
HS7	-22CKYasp27-	-30ateFRA35-			-94CKIelm98-		-121cpdDQE-
HS8	-22CKYasp27-	-30ateFRA35-			-94CKIelm98-	-111tqiHVK118-	-123CPSDQE-
HS9	-22CKYasp27-	-30ateFRA35-				-113TIIyvi116-	-121cpdDQE-
HS10			-47VCVaty53-	-56gneLQV60-	-94CKIelm98-		-121cpdDQE-
HS11	-22CKYasp27-	-30ateFRA35-	-47VCVaty53-	-56gneLQV60-	-94CKIelm98-		-121cpdDQE-
HS12			-47VCVaty53-	-56gneLQV60-	-94CKLelm98-	-111tqiHVK118-	-123CPSDQE-
HS13	-22CKYasp27-	-30ateFRA35-	-47VCVaty53-	-56gneLQV60-	-94CKIelm98-	-111tqiHVK118-	-123CPSDQE-
HS14			-47VCVaty53-	-56gneLQV60-			-123CPSDQE-

Junction sequences of the CTLA4 /CD28-Ig hybrid fusion proteins. Amino acids are denoted by their single letter code with those in upper case being CD28 residues, those in lower case being CTLA4 residues and those in bold upper case being human IgG1 residues. Numbering is from the mature N-terminal of the respective proteins and refer to the adjacent amino acid in the table.

TABLE II. Binding of CTLA4 and CD28 monoclonal antibodies to CTLA4Ig and CD28Ig mutant fusion proteins and to CTLA4/CD28Ig hybrid fusion proteins.

	anti-CTLA4 mAbs			anti-CD28 mAb
	7F8	11D4	10A8	9.3
CTLA4Ig MUTANT FUSION PROTEIN				
AYPPPY	+++	+++	+++	-
MAPPPY	++	+	++	-
MYAPPY	+	-	+	-
MYPAPY	+++	+++	+++	-
MYPPAY	+++	-	+	-
MYPPPA	+++	++	+++	-
AAPPPY	+	++		
CD28Ig MUTANT FUSION PROTEIN				
MYPPAY	-	-	-	-
MYPPPA	-	-	-	+
CTLA4/CD28Ig HYBRID FUSION PROTEINS				
HS1	-	-	-	-
HS2	-	-	-	+
HS3	-	-	-	-
HS4	-	-	-	+++
HS5	-	-	-	-
HS6	+	-	-	-
HS4-A	-	-	-	++
HS4-B	-	-	-	++
HS7	-	-	-	+++
HS8	-	+	-	+++
HS9	-	+	-	-
HS10	-	-	-	-
HS11	-	-	-	+
HS12	-	-	-	-
HS13	-	-	-	-
HS14	-	-	-	-
CTLA4Ig	+++	+++	+++	-
CD28Ig	-	-	-	+++

Antibody binding was rated from that seen for wild type protein (+++) to above background (+), and no detectable binding (-).